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THE EFFECT OF SEASON UPON THE LIFE CYCLE OF

HAEMONCHUS CONTORTUS IN EXPERIMENTALLY

INFECTED LAMBS

BY

MARISSA BRUMMETT

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

BIOLOGICAL AND ENVIRONMENTAL SCIENCE

UNIVERSITY OF RHODE ISLAND

2019

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MASTER OF SCIENCE THESIS

OF

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2019

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ABSTRACT

This project was developed to study the life cycle of the most pathogenic gastrointestinal nematode (GIN) affecting small ruminants, *Haemonchus contortus*. The main goal of the study was to test the effects that the environment and the age of the adult worm have on the resulting egg's ability to hatch *in vitro* and the larvae's ability to undergo exsheathment both artificially *in vitro* as well as *in vivo* within the rumen. A secondary study objective was to compare the results from the two exsheathment assays in order to see how well they correlate.

This study was designed to run in a series of cycles according to the start of each season (cycles denoted as: Fall 1, Winter, Spring, Summer and Fall 2). Each cycle began with the infection of two genetically related donor lambs with *H. contortus* larvae (L3). Larvae and eggs were harvested from the donor lambs for 4-6 months (increasing worm ages) for each cycle and were subjected to *in vitro* egg hatch and *in vitro* and *in vivo* exsheathment assays, respectively. Eggs were incubated in a well plate at 26°C and after 24 hours of incubation, hatchability of eggs was assessed under the microscope. For the *in vitro* exsheathment assay, larvae were bubbled with CO_2 for 15 minutes and incubated at 37°C for 18 hours and were then observed under the microscope to determine percent live exsheathment, as well as viability. For the *in vivo* assay, larvae were added to a containment capsule and suspended in the rumen of four fistulated ewes for 8 hours, recovered and examined under the microscope to determine percent live exsheathment, as well as viability. Data collected from the two exsheathment assays were statistically compared to one another according to seasonal effects and age of worm effects.

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It was found that neither season nor age of worm had any effect on egg hatchability. There was a season*worm age effect on both *in vitro* and *in vivo* exsheathment and viability across multiple seasons and worm ages tested. Increased variability in *in vitro* exsheathment rates was detected during the Fall 1, Summer and Fall 2 cycles, with lower variability during the Winter and Spring cycles. Upon comparing the results of the two assays, it was determined that the *in vitro* assay yielded higher viability rates, but lower and more variable exsheathment rates when compared to the *in vivo* assay.

The results of this study indicate that both season and age of worm have an impact on exsheathment of *H. contortus* larvae both *in vitro* and *in vivo*, especially during the Fall and Summer seasons. The findings of this study also indicate that the *in vitro* and *in vivo* assay yield variable results according to different seasons and worm ages. This study has shed light on the role that both season and age of worm play in the exsheathment stage of the *H. contortus* life cycle, indicating that these factors need to be studied in more depth in future research. Additionally, more work should be done to compare different *in vitro* and *in vivo* exsheathment assays in order to refine methodology for how *H. contortus* is studied in anthelmintic research.

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PREFACE

This thesis has been prepared using the Manuscript Format. Chapter I contains a literature review, while Chapter II contains a manuscript that will be submitted for publication. Chapter III covers a summary of future research directions.

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CHAPTER I
REVIEW OF LITERATURE

1. Gastrointestinal Nematode Infection in Small Ruminants

1.1 Overview

Gastrointestinal nematode (GIN) infection poses a major threat to the health of small ruminants. Infections have been identified as a major limitation for pasture-based production of small ruminants resulting in significant production and economic losses for producers (Hoste et al., 2006). Small ruminants suffering from GIN infection may experience severe anemia, weight loss, diarrhea and in acute cases, death, especially in lambs and pregnant females, as they are more susceptible to infection (Santos et al., 2014).

1.2 Economic Impact and Production Loss

The FAO (Food and Agriculture Organization of the United Nations), reported that a total of 567.7 sheep (producing and animals slaughter) existed in world in 2017 (FAOstat, 2017). The FAO also reported the worldwide sheep production value to be 40,382 million dollars in 2016 (FAOstat, 2016). The 2017 USDA Census of Agriculture reported that a total of 237,829 sheep and goat farms were operating in the United States (USDA, 2017) showing an increase from the 2012 census (USDA, 2012). Sales in the United States for sheep and goat agriculture—including meat production and secondary products such as wool and mohair—totaled 923.6 million dollars

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(USDA, 2017). According to the 2015 United States Department of Agriculture Census of Agriculture, 21,239 lambs (9.1% nonpredator death losses) and 13,543 sheep (8.6% nonpredator death losses) were lost due to internal parasites (USDA, 2015).

1.3 Anthelmintic Resistance

Due to overuse of commercial dewormers, anthelmintic resistance has emerged as a leading issue associated with gastrointestinal nematode infection and has become an issue of global concern (Assis et al., 2003; Hoste et al., 2006; Kaplan and Vidyashankar, 2012). Anthelmintic resistance in sheep parasites was first reported in the 1950s and 1960s (Kaplan, 2004) and by 1990, the issue had become a worldwide threat to small ruminant production (Waller, 1999). Studies have been conducted across the US to document patterns of resistance. Mortensen et al. (2003) reported that resistance to ivermectin and albendazole was prevalent on 18 out of 19 goat farms in Georgia in 2003, while there was no prevalence of moxidectin resistance. A follow-up regional study conducted during 2004–2006 detected resistance to moxidectin on 24% of sheep and goat farms surveyed (Howell et al., 2008), which suggests a rapid evolution of moxidectin resistance in GIN species with a background of ivermectin resistance (Terril et al., 2012). Within the last 11 years, two new drug classes have been introduced, monepantel and a combination of derquantel and abamectin, but recent work has confirmed cases of monepantel resistance in New Zealand (Kaminsky et al., 2008; Little et al., 2010; Van den Brom et al., 2016). It has been proposed that new classes of anthelmintics alone will not solve the issue of anthelmintic resistance. By developing and altering strategies, producers can better preserve the efficacy of existing and future anthelmintic products (Kaplan and Vindyashankar, 2012).

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Widespread anthelmintic resistance has created a need for research and development of anthelmintic alternatives. One promising area of anthelmintic research focuses on plants that contain condensed tannins and other plant secondary compounds. Forages containing these compounds, such as sericea lespedeza (*Lespedeza cuneata*), have been shown to have an anthelmintic effect against different life stages of GIN species (Lange et al., 2006; Shaik et al., 2006). Other tannin-containing forages, such as birdsfoot trefoil (*Lotus corniculatus*) and cranberry vine, have been investigated in anthelmintic research involving the economically significant GIN species, *Haemonchus contortus* (Heckendorn et al., 2007; Barone et al., 2018; Barone et al., 2019). Using various *in vitro* and *in vivo* assays, it has been observed that condensed tannins have an inhibitory effect on the hatchability of eggs produced as well as on the exsheathment process of L3 stage larvae in *H. contortus* (Acharya et al., 2014; Alonso-Díaz et al., 2008; Brunet et al., 2007).

2. *Haemonchus Contortus*

2.1 Overview

Haemonchus contortus is the most pathogenic GIN species infecting small ruminants across the world (Verissimo et al., 2012). This parasite is known to cause severe anemia due to its hematophagous tendencies, which lead to hemorrhage within the abomasum, ultimately killing heavily infected animals (Acevedo-Ramirez et al., 2019). Fatality is most common in lambs due to an underdeveloped immune system (Besier, 2012), as well as pregnant females due to a suppressed immune system resulting from metabolic demands of pregnancy (Vlassoff et. al., 2001).

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2.2 Haemonchus Contortus Life Cycle

Haemonchus contortus is a trichostrongyle nematode that matures and reproduces in the abomasum, where it feeds on the host's blood, causing anemia in susceptible animals (Ortolani et al., 2013; Emery et al., 2016). The life cycle of this parasite is depicted in **Figure 1**. Adults mate and produce eggs that are shed in feces (Roeber et al., 2013). The eggs hatch yielding larvae that go through the L1 and L2 stages within the feces, emerging from the fecal pellet as infective third stage larvae (L3), which are consumed by grazing animals. The L3 larva is encased in a protective sheath covering that must be shed in order for the larva to develop into L4 stage. The process of shedding the sheath occurs in the rumen and is termed 'exsheathment.' The exsheathment process is critical for establishment of infection within the host. Although the details are not fully understood, it is believed that carbon dioxide (CO_2) within the rumen environment plays a role in inducing exsheathment (Nikolaou & Gasser, 2006). Once exsheathed, the larvae move to the abomasum where they continue to mature through L4 and L5 stages, becoming adult blood sucking worms (Roeber et al., 2013). It has been previously determined that the rate at which the larvae mature can vary according to age and susceptibility of the host animal, with larvae maturing faster in younger or more susceptible animals (Silverman and Patterson, 1960).

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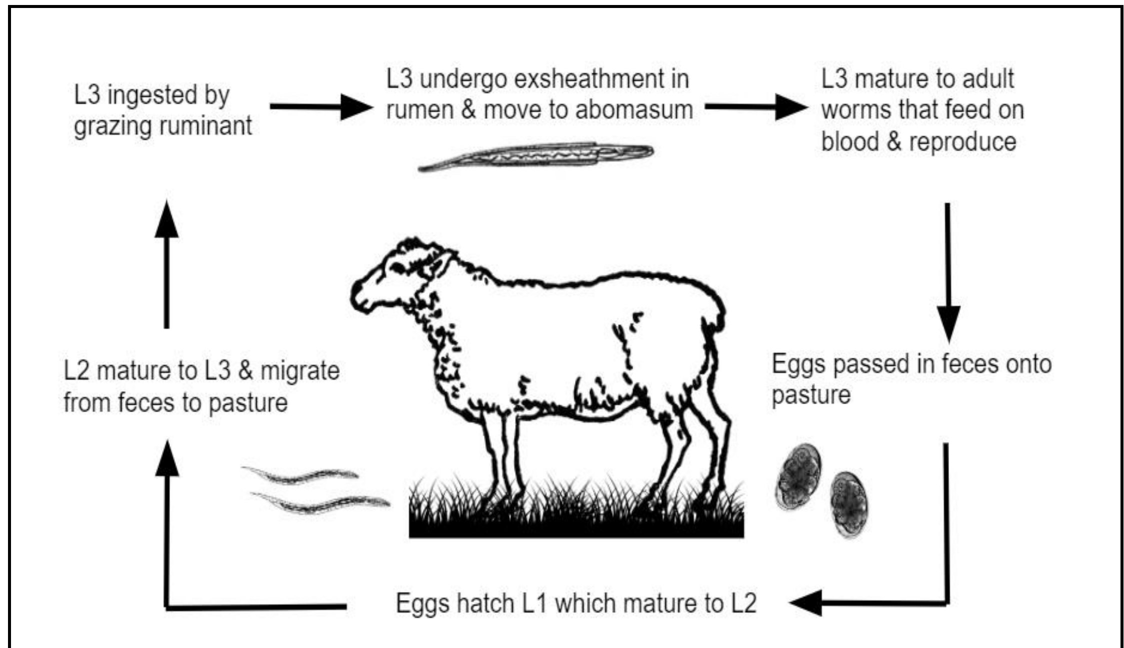

Figure 1. *The life cycle of Haemonchus contortus in sheep.*

2.3 Experimentally infected animals used in Haemonchus contortus research

Young animals are commonly used in research involving *H. contortus*. Lambs are typically experimentally infected in order to be used as “donor lambs” for eggs and/or larvae to be harvested for *in vitro* work, or they are infected to be used in various in *vivo* studies. The age of lambs used varies according to specific study objectives, ranging from 2 to 10 months old (Katiki et al., 2012; Zajac et al., 1990). It has been previously suggested that age of host can influence larval development in trichostrongyle infection. Mature ewes demonstrate a reduced development of larvae to L3 stage when compared to development patterns in 3-month-old lambs (Jorgensen et al., 1998). This supports using lambs as donor animals, as opposed to using mature animals.

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2.3.1 Immune status in lambs

Hohenhaus et al. (1995) investigated immune responsiveness in lambs of different ages, ranging from 4 months to 15 months old (Hohenhaus et al., 1995). They found that antibody levels against *H. contortus* and *T. colubriformis* increased significantly going from lambs 6 months of age to lambs 9 months of age, but between 12 and 15 months of age, these changes were not apparent (Hohenhaus et al., 1995). This suggests that by 12 months of age, antibody titers may reach a maximum/steady state. It has been previously determined that host immunity can have an impact on fecundity of adult female nematodes (*Teladorsagia circumcincta*) in sheep (Strain et al. 2002).

Lamb age has been shown to affect vaccine efficacy. Kooyman et al. (2001) found that lambs aged 6 and 9 months of age were 77% and 83% protected against *H. contortus* infection, respectively, whereas 3-month-old lambs showed no sign of protection after receiving the vaccine, which could be due to maternal antibody clearance. They also found a higher IgE serum level, higher eosinophil count and mucosal mast cell hyperplasia in older, vaccinated sheep (typical of a Th2 immune response) when compared to younger, unprotected lambs. These differences may be related to inability of immature immune systems in younger lambs to induce IgE responses (Kooyman et al., 2001).

2.3.2 Gender as a factor for infection development

Male lambs are more commonly used as donor animals in studies involving experimental infections due to greater ease of large quantity fecal sample recovery. Several studies have demonstrated that male lambs are generally more susceptible than

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females to nematode infection (Barger, 1993; Luffau et al., 1981; Adams, 1989). Male lambs develop a lower immunity to infection when compared to female lambs (Luffau et al., 1981), and male lambs develops higher worm burdens, with ewes demonstrating worm burdens only 40% as high as males (Adams, 1989).

Although males tend to show lower immunity and/or higher susceptibility to *H. contortus* infection, it is not guaranteed that males will always develop infection differently from females. Several studies have found there to be no consistent differences between males and females for host susceptibility to *H. contortus* (Albers et al., 1989; Woollaston et al., 1990). One particular study even reported that males on trial had significantly lower fecal egg counts than females on trial, and the researchers noted that this finding contradicts findings from similar studies (Shaw et al., 1995).

3. *Haemonchus contortus* Life Cycle Stage: Egg Hatch

3.1 Egg Hatch Stage

One adult female worm can lay between 5,000 and 15,000 eggs per day (Emery et al., 2016), allowing animals with heavy worm burdens to deposit millions of eggs on pasture. Hatching of *H. contortus* eggs and release of L1 larvae are proposed to be the result of both mechanical and enzymatic events which are not completely understood (Rogers and Brooks, 1977; Bone and Parish, 1988; Perry, 1989). It has been proposed that abomasal pH can have an effect on egg laying-- increasing acidity results in higher egg production (Honde and Bueno, 1982). The shell of *Haemonchus contortus* eggs is composed of three layers: an outer vitellin layer, a middle, chitinous layer and inner phospholipid layer (Mansfield et al. 1992). In a study where *H. contortus* eggs were exposed to proteases, all three layers of the shell were disrupted,

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indicating that the layers are primarily composed of protein (Mansfield et al., 1992). Studies have also found that the eggs of *H. contortus* contain particular ABC transporter, P-glycoprotein (Pgp), which plays a role in mechanisms for anthelmintic resistance (Kerboeuf et al. 2003).

The egg hatch stage of *H. contortus* is commonly studied in anthelmintic research (Fouche et al., 2016; Varady et al., 2007; Hoekstra et al., 1997). Many studies investigate the effect of acetone: water extracts from tannin-rich plants on egg hatching (Chan-Perez et al., 2016; Fouche et al., 2016). One particular study that looked at multiple types of tannin containing forages found that four different foliage extracts-- L*ysiloma latisiliquum*, *Laguncularia racemosa*, *Avicenna germinans* and *Theobroma cacao*-- showed anthelmintic activity specifically by inhibiting the 'eclosion' or hatching of *H. contortus* eggs in the *in vitro* egg hatch assay (Vargas-magana et al., 2014). A limited number of studies have examined the direct mechanism of action of tannins on nematode egg hatching (Vargas-magana et al., 2014). Condensed tannins are not absorbed from the gastrointestinal tract of ruminants (Terrill et al., 1994) and so it has been predicted that the tannins are excreted with the eggs in feces and act as inhibitors of egg hatching-- a theory that has been suggested by multiple *in vitro* studies (Molan et al., 2002, Min and Hart, 2003, Molan and Faraj, 2010)

3.2 Egg Hatch Assay

There are multiple variations of the standard procedure for conducting *in vitro* egg hatch assays (Coles et al., 1992; Assis et al. 2003; Marie-Magdeleine et al. 2009; Barone et al., 2018). The egg hatch test (EHT) proposed by Coles et al., has been used to detect benzimidazole resistance in different strains of *H. contortus* larvae (Coles et

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al., 2006; Varady et al., 2007). The egg hatch test proposed by Assis et al., (2003) was a modified version of the original method (Coles et al., 1992), which was further modified by Marie-Magdeleine et al., (2009) and is referred to as the egg hatch assay. The egg hatch assay involves incubation of *H. contortus* eggs in a PBS buffer in a well plate at 25 °C for 48 hours (Marie-Magdeleine et al., 2009). The well plate is observed under a microscope and larvae and eggs are counted to determine percent hatchability. Both the egg hatch test and the modified egg hatch assay have been used in numerous studies to assess anthelmintic activity of tannin rich forages (Adamu et al., 2013; Fouche et al., 2016; Barone et al., 2018).

4. *Haemonchus Contortus* Life Cycle Stage: Exsheathment

4.1 Mechanism of Exsheathment

One of the most crucial life stages for establishment of *H. contortus* infection is 'exsheathment'. The exsheathment stage is essential for the transition from free living stages to parasitic stages within the *H. contortus* life cycle (Hertzberg et al., 2002). During exsheathment third stage larvae (L3) shed an outer cuticle covering; this takes place in the rumen of the animal (Sommerville, 1957). The cuticle covering serves mainly to protect the larva from the environment during the free-living stages of the life cycle and can help to prevent degradation by proteases (Rhoades and Fetterer, 1996). Previous *in vitro* studies have been influential in developing the current hypothesis for the mechanical/chemical mechanism of larval exsheathment, and are summarized in a review article by Nikolaou and Gasser (2006). Although the exact mechanism is not fully understood, it is widely accepted that exsheathment is triggered by CO_2 within the

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rumen compartment of the ruminant stomach (Nikolaou & Gasser, 2006). Rogers and Sommerville (1960) were able to determine that rumen fluid induces exsheathment, but once stimulated, rumen fluid could be removed and exsheathment would still occur. They also discovered that temperature, pH and oxidation-reduction potential of rumen fluid influenced stimulation of larval exsheathment (Rogers and Sommerville, 1960). It has been suggested that the enzyme, carbonic anhydrase plays a role in inducing exsheathment (Wharton, 1991). The anterior portion of the cuticle contains chemoreceptors that sense carbonic anhydrase in the rumen environment, which then triggers the release of noradrenaline, further activating the actual process of exsheathment (Nikolaou & Gasser, 2006). Larvae release an exsheathing fluid-- thought to be released from an excretory pore orifice located between the nerve ring and base of the esophagus-- which erodes the sheath (Sommerville, 1957; Rogers and Sommerville, 1960). The release of the fluid causes a refractile ring to develop, which leads to a break in the sheath, creating a cap end that opens and allows the larvae to maneuver out of the sheath (Rogers and Sommerville, 1960; Wharton, 1991). The process of exsheathment is depicted in Figure 2. Once exsheathed, the infective L3 are able to mature through the L4 and L5 stages before becoming adult blood sucking worms.

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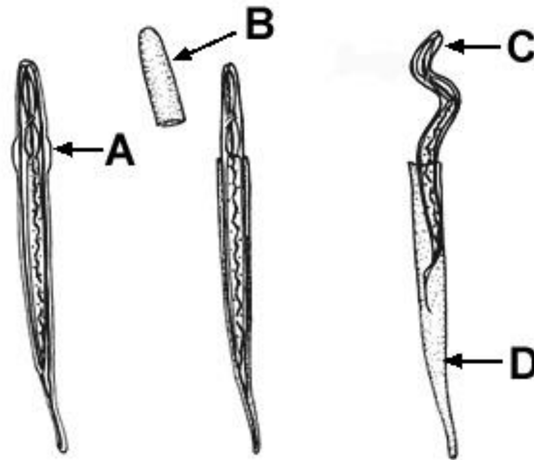

Figure 2. *Haemonchus contortus larval exsheathment*. The ensheathed L3 is triggered by CO_2 to release enzymes and exsheathing fluid, which weaken the cuticle (A). The cuticle breaks to form a cap (B) portion and the exsheathed L3 (C) escapes its sheath (D). (Parasites and Parasitic Diseases of Domestic Animals- Dr. Colin Johnstone http://cal.vet.upenn.edu/projects/merial/Nematodes/nems_7.htm

4.2 In vitro Exsheathment Assays

Multiple assays have been developed to induce larval exsheathment *in vitro*. One of the most common exsheathment assays that is used in anthelmintic research is the bleach assay, which the uses a solution of sodium hypochlorite (2% w/v) to induce exsheathment (Conder and Johnson, 1996; Bahuaud et al., 2006). In this assay, *H. contortus* larvae are typically incubated in combination with tannin containing plant

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extracts for three hours before being rinsed with phosphate buffer solution (PBS) (Bahuaud et al., 2006). After rinsing, the larvae are subjected to a sodium hypochlorite solution (2% w/v) and exsheathment is analyzed under a microscope every 10 minutes for a period of one hour (Bahuaud et al., 2006). Since its initial development, the larval exsheathment bleach assay has been used in many studies that focus on testing various tanniferous plant species for anthelmintic properties (Alonso-Diaz et al., 2008; Son-de Fernex et al., 2012; Araújo et al., 2017).

A different *in vitro* exsheathment involving the use of CO_2 to induce exsheathment in *H. contortus* L3 [assay was developed by Conder and Johnson in 1996]. In the CO_2 exsheathment assay, L3 larvae are added to a polypropylene tube with 10 ml of Earle's Balanced Salt Solution. The tube of larvae is covered with Parafilm® and CO_2 is bubbled into the solution for 10 minutes using a glass pipet tip attached to CO_2 tank inserted through the parafilm (Conder and Johnson, 1996). The tube is then capped and incubated at 37° C for 18 hours. Exsheathment counts are determined post-incubation by observing 100 live larvae under a microscope (Conder and Johnson, 1996). This method yields exsheathment rates ≥ 93.5% and larvae subjected to this treatment show higher infectivity rates than larvae subjected to treatment using sodium hypochlorite (Conder and Johnson, 1996). The CO_2 method has been used in a few recent studies, but is not as commonly used as the bleach method proposed by Bahuaud et al. (2006) (Barone et al., 2018; Barone et al., 2019).

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4.3 In vivo exsheathment assays

While exsheathment of *H. contortus* L3 has been widely studied using *in vitro* methods, only a few studies have used *in vivo* methods (Sommerville, 1957; Hertzberg et al., 2002; Brunet et al., 2007; Lonngren et al., 2017). These studies varied in the larval containers used for suspension in fistulated animals, as well as in the time periods for exsheathment incubation (Sommerville et al., 1957; Hertzberg et al., 2002; Brunet et al., 2007). There are a number of issues associated with the methodology proposed in previous studies including issues with protocol supplies, larval containment issues and consistency of exsheathment results due to inability of rumen fluid to effectively flow through larval capsules and due to these inconsistencies, a new protocol for evaluating larval exsheathment *in vivo* was developed by Lonngren et al. (2017). After testing three different types of capsules, Lonngren et al. (2017) chose one composed of a piece of Tygon tubing and Nunc™ Cell Culture Inserts, a design which resembled the capsules used by Brunet et al. (2007). It was determined that larvae exsheathed consistently at an average rate of $82 \pm 1\%$ during an 8-hour exposure period (Longren et al., 2019). The results produced by the 8-hour exposure using Nunc™ capsules are most consistent with results published by Sommerville (1957) but vary significantly from other findings (Hertzberg et al., 2002; Brunet et al., 2007). Variability in larval performance for both *in vivo* and *in vitro* exsheathment assays was observed by Lonngren et al. (2017). In their experiments, fecal matter was cultured at room temperature for 8 days, which can potentially have an influence on exsheathment of the resulting L3 larvae. It is also possible that larvae harvested from older donor infections may not exsheath as well due to the age of the adult parasites producing the larvae

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(Lonngren et al., 2017). It is possible that environmental conditions may also have an impact on larval performance in exsheathment assays, but time of year/season is not mentioned in previous *in vivo* studies (Sommerville, 1957; Brunet et al., 2007; Hertzberg et al., 2002 Lonngren et al. 2017). The variability of *in vivo* exsheathment data, as well as inconsistencies observed between different batches of *H. contortus* L3 larvae indicate a need for further exploration of potential factors influencing exsheathment.

5. Temperature and Environmental Impacts on *Haemonchus contortus* Life Cycle

5.1 Hypobiosis of H. contortus

It is known that during the L4 stage of their life cycle, *H. contortus* are able to transition into a hypobiotic state of arrested development (Gatongi et al., 1998). It was initially hypothesized that hypobiosis was a result of host resistance to infection, but further research has confirmed that environmental factors play a crucial role in triggering this phenomenon (Waller and Thomas, 1975; Eysker, 1981; Gatongi et al., 1998).

5.1.1 Hypobiosis in temperate climates

In temperate climates, hypobiosis allows for “overwintering” of *H. contortus* within the host, allowing the larvae to survive the cold winter months and resume development once the environment becomes favorable again in the spring (Gatongi et al., 1998; Blitz and Gibbs, 1972; Waller et al., 2004). Studies exploring patterns of hypobiosis have been performed in different temperate regions including England,

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Central Europe, Canada, New Zealand and the United States (Waller and Thomas, 1975; Langrova et al., 2008; Falzon et al., 2014; McKenna, 1974; Blitz and Gibbs, 1972; Zajac et al., 1988). In the United States, it has been suggested that decreasing temperature and photoperiod are associated with onset of hypobiosis and may be crucial stimuli for the free-living stages of this nematode species (Capitini et al., 1990; Langrova et al., 2008). This concept has little support from studies performed in other countries with a similar temperate climate, with evidence of hypobiosis occurring as early as August in England and Quebec, Canada (Connan, 1971; Blitz and Gibbs, 1972).

Source of L3 (culture and storage conditions) used to infect study subjects has been identified as a potential factor influencing variability in observed hypobiosis patterns (Capitini et al., 1990). In studies in both England and Canada that used freshly cultured larvae for infection of lambs before slaughter and larvae/worm collection, researchers observed levels of hypobiosis ranging from >90% to 20-45%, respectively (Connan, 1975; Blitz and Gibbs, 1972). Results from studies in The Netherlands and New Zealand using larvae in different storage conditions (5-21°C for 30-80 days) all yielded high levels of hypobiosis in specific *H. contortus* strains (Eysker, 1981; McKenna, 1973). These patterns were explored in depth in a study performed in Ohio by Capitini et al., (1990). The researchers performed two different experiments-- one focusing on autumn temperature conditions and one on summer temperature conditions-- that tested 17 different larval conditioning methods with varying temperature, photoperiod simulations and culture conditions using a known strain of *H.contortus* that had previously exhibited 98-100% hypobiosis in naturally infected

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sheep during winter conditions (Capitini et al., 1990; Herd et al., 1984). The researchers used a total of 108 crossbred 3-month-old lambs, divided into different treatment groups that were infected using larvae conditioned by different methods using environmental chambers and refrigerators (Capitini et al., 1990). The researchers found that across the different larval conditioning procedures of varying temperatures and photoperiod treatments, overall incidence of hypobiosis was low, ranging from 0-36% (Capitini et al., 1990). It was also determined that there were no significant differences in level of hypobiosis across the different treatment groups when compared to the control group (untreated *H. contortus* derived from fresh culture) (Capitini et al., 1990). The highest incidence of hypobiosis for the autumn experiment was observed for three treatment groups that had all been given conditioned L3 larvae exposed to 20°C followed by sudden or gradual decrease to 4°C. In the summer experiment, hypobiosis was observed for 4 out of 9 treatment groups, but only at low intensities ranging from 1.2-4.1% (Capitini et al., 1990). The researchers noted that although results from the study showed variation and did not agree with findings from previous work, another United States study by Mansfield et al., (1977) also failed to induce hypobiosis in larvae using different culture/storage temperature conditions. The researchers concluded that a possible explanation for the results of their study, as well as overall variability observed across different studies, is that seasonal hypobiosis of *H. contortus* is an obligatory genetic strategy that occurs without direct external stimuli (Capitini et al., 1990). Differences between strains of *H. contortus* further supports the authors' hypothesis that hypobiosis occurs without direct external stimuli.

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5.1.2 Hypobiosis in tropical climates

In regions with a tropical climate, such as Africa, hypobiosis of *H. contortus* occurs during the "dry season", when conditions are unfavorable for larval development (Fakae, 1990). This stage of arrested development ends at the beginning of the "rainy season", as *H. contortus* larvae are able to resume development in wet conditions (Okon and Enyenihi, 1975; Fakae, 1990). Studies exploring hypobiosis in different tropical areas of the world have yielded variable results, depending on specific study sites (Gatongi et al., 1998). For example, studies performed in countries such as Egypt and Brazil have reported no evidence of hypobiosis, whereas in Zimbabwe and Mauritania, low levels of hypobiosis have been reported and in Nigeria, high levels of hypobiosis were detected (Charles, 1989; El-Azazy, 1990; Pandey et al., 1994; Jacquiet et al., 1995; Ogunsusi and Eysker, 1979).

It has been suggested that there are two "modes" for occurrence of hypobiosis in *Haemonchus contortus* (Gatongi et al., 1998). The first mode is predominantly observed in temperate climates and is characterized by a parasite population within the host that is composed exclusively of larvae in the hypobiotic state (Blitz and Gibbs, 1972; Waller and Thomas, 1975). The second mode is predominantly observed in tropical climates and is characterized by a parasite population within the host that is composed of larvae in the hypobiotic state coexisting with adult worms (Ogunsusi and Eysker, 1979; Pandey et al., 1994; Jacquiet et al., 1995). Evidence for the second mode has led to the hypothesis that in some tropical areas, hypobiosis is not essential for *H. contortus* survival (Gatongi et al., 1998). Studies have shown that levels of hypobiosis increase during the dry season and decrease during the wet season whereas levels of

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adult worms decrease during the dry season and increase during the wet season (Pandey et al., 1994; Gatongi et al., 1998). This evidence suggests that although it may not be exclusively responsible for survivability of *H. contortus* infection, hypobiosis is still considered to be advantageous for this species (Gatongi et al., 1998).

5.2 Effect of Temperature on Egg Hatch

It is common practice to store fecal samples containing nematode eggs in a refrigerator (4°C) in order to prevent egg hatching before fecal egg counts and/or other assays are performed (Crofton and Whitlock, 1965; Foreyt, 1986; Hertzberg et al., 2002; Zajac and Conboy, 2012). Different studies have examined the effects of cold storage/incubation on *H. contortus* egg survivability and hatchability. Smith-Buijs and Borgsteede (1986) reported that incubation at 4°C for a period of one week is lethal for *H. contortus* eggs. Jasmer et al. (1986) examined both the ability of *H. contortus* eggs to develop when incubated at 10°C and the hatchability of eggs when exposed to temperatures of -18°C. They found that after 5 days of incubation, only 32% of *H. contortus* eggs had developed to the larvated stage, whereas for *Ostertagia circumcincta*, 95% of incubated eggs had developed to the larvated stage. When exposed to -18°C, *H. contortus* eggs exhibited <4% survivability (i.e. hatchability), compared to >87% for *O. circumcincta* (Jasmer et al., 1986).

Mckenna (1998) aimed to investigated how length of exposure time to cold temperature (4°C) influenced success rate in development to third stage larvae. Fecal samples were incubated at 4°C for different periods of time (0, 1, 3, 7 and 12 days) and were then transferred to an incubator at 27°C for 7 days to allow hatching of eggs and development to third stage larvae to occur. Both 7- and 12-day incubation periods

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produced significantly lower L3 larval yields (P <0.05) when compared to 0- and 1-day incubation periods. The L3 larval yield for the 12-day incubation period was also significantly lower (<0.05) than that of the 3-day incubation period (Mckenna, 1998). Viability of *H. contortus* eggs decreased from 100% to 0% going from 0 days to 12 days of cold storage, showing a sharp decrease after 3 days of incubation (Mckenna, 1998). To date no studies have specifically examined the effect of natural seasonal changes (as opposed to experimental) on *H. contortus* egg hatch *in vitro.*

5.3 Effect of Temperature on Haemonchus contortus L3 larvae

5.3.1 Effect of cold temperature incubation/storage on L3 larvae

There are two main methods for storing *H. contortus* L3 larvae--cryopreservation and refrigerator storage (Chylinski et al., 2015). In cryopreservation, L3 are exsheathed and placed in physiological serum and stored in liquid nitrogen (Van Wyk et al., 1977). Although this technique yields viable larvae that remain infective to sheep after 10 years of storage, it is not useful for storage of larvae to be used in exsheathment assays, as the larvae are exsheathed prior to storage (Rew and Campbell, 1983; Campbell et al., 1973). The refrigerator storage technique allows stocks of L3 larvae to be maintained at 4°C for several months (MAFF, 1986). Sheathed larvae are unable to feed and rely on energy reserves while in storage (O'Conner et al., 2006; Chylinski et al., 2015). Cool storage works by reducing larval metabolic rate, slowing depletion of energy stores (Vlassoff et al., 2001).

Refrigerator storage of *H. contortus* L3 larvae is well documented, with most published studies noting that larvae used in their studies had not been in storage for more than three months (Hertzberg et al., 2002; Brunet et al., 2007). Chylinski et al.

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(2015) investigated the effect of long-term refrigerator storage on the establishment of infection and the egg to L3 larvae development ratio. They found that for a particular strain of *H. contortus* both infection establishment (number of adults) and ability of eggs to develop to the L3 stage were significantly reduced ($p < 0.05$) in larvae stored for 16 months compared to larvae stored for 4 months (Chylinski et al., 2015). In their study, they did not find any significant differences for any life cycle parameters for larvae stored for 7 months when compared to that stored for 4 months. The researchers noted that these findings are drastically different from the effects of storage on *Trichostrongylus retortaeformis*, where negative effects were observed after only 9 weeks in storage (Kerboeuf, 1978; Mallet and Kerboeuf, 1985). The findings of this study also do not agree with the common recommendation (< 3 months) for refrigerator larvae storage.

5.3.2 Effects of cold storage on exsheathment stage of L3 larvae

Larvae used in exsheathment assays are commonly stored using the refrigerator storage method, and so it is important to understand how cold storage can impact the ability of the larvae to exsheath. Early studies exploring the effects of cold storage on larval exsheathment yielded variable results. Silverman and Podger (1964) noted that there were no observed differences in exsheathment rates for larvae stored at 4°C for 1 week and 3 months, however the exsheathment methodology in the study is unclear. Slocombe and Whitlock (1970) investigated the role of time in cold storage as a factor influencing *in vitro* exsheathment. Larvae were stored for varying amounts of time (1-12 weeks) at 4°C before being subjected to *in vitro* exsheathment. Exsheathment was induced by combining larvae with 0.02 M- $Na_2B_4O_7.10H_2O$ into which a gas mixture of

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40 % CO_2-60 % N_2 was bubbled for 3 minutes followed by either a 20 minute or 60-minute incubation period (Slocombe and Whitlock, 1970). No significant differences were observed for exsheathment of unstored L3 larvae compared to larvae stored for 1-12 weeks for the 60-minute incubation period. A gradual decrease in exsheathment rates was observed for the larvae subjected to the 20-minute incubation period going from 1-12 weeks of cold storage (Slocombe and Whitlock, 1970). For all 20-minute incubation experiments and some 60-minute incubation experiments, the researchers observed a notable decrease in exsheathment rates 24 hours after the larvae were placed in cold storage. This was suggested to be the result of cold shock, which the larvae recovered from after four days (Slocombe and Whitlock, 1970).

5.3.3 Effects of high temperature incubation and heat shock on L3 larval exsheathment

In the GIN species, *Trichostrongylus axei* and *Labiostrongylus eugenii*, it has been shown that an increase from ambient temperature to temperature that replicates that of the host's body plays a role in triggering the exsheathment process (Campbell and Gaugler, 1991; Smales and Sommerville, 1977). Early work has shown that temperature specifically impacts exsheathment by influencing the formation of the refractile ring in certain abomasal species, including *T. axei* and *O. circumcincta* (Rogers and Sommerville, 1960). It was determined that a temperature of 40°C was optimal for refractile ring formation in these species. Furthermore, it was shown that for both species, an elevated temperature was needed to trigger exsheathment, but exsheathment was not inhibited by subsequent reduction in temperature (Rogers and Sommerville, 1960).

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Bekelaar et al. (2018) investigated how the rate of change in temperature and the magnitude of change in temperature each impacted exsheathment both in the presence and absence of CO_2. The researchers determined that larvae exposed to heat shock (rapid change in temperature to a threshold of 35-40°C) exhibited higher rates of exsheathment when compared to larvae exposed to a constant or gradually elevated temperature (Bekelaar et al., 2018). They determined that heat shock (in combination with CO_2 exposure) induced exsheathment, but exsheathment rates were not inhibited by a subsequent decrease in temperature to 19°C. This finding implies that heat shock acts as a trigger for the exsheathment but is not required during the actual process of exsheathment (Bekelaar et al., 2018). The study also found that a minimum final temperature (post heat shock) of 30°C was needed for exsheathment to occur and that maximal exsheathment rates were observed at final temperatures over 40°C (Bekelaar et al., 2018). Only a small percentage of L3 larvae exhibited exsheathment when exposed to gradually increasing temperatures even in the presence of CO_2 (Bekelaar et al., 2018). In order to reach 50% exsheathment, L3 larvae had to be continuously exposed to temperatures of 40°C for at least 3 days. These findings further support the notion that heat shock is more crucial as a biological factor for inducing exsheathment, than temperature itself (Bekelaar et al., 2018). A follow up study tested this hypothesis using rumen fluid as a medium combined with CO_2 and heat shock (Bekelaar et al., 2019). The results yielded >80% exsheathment in under 4 hours for *H. contortus* (Bekelaar et al., 2019). Future work should focus on exploring the specific biological pathways triggered by heat shock, as well as additional host or environmental factors

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that may influence the rate of exsheathment when the magnitude of heat shock is smaller (Bekelaar et al., 2018).

5.4 Effects of Climate on H. contortus development

With the rising issue of climate change, recent studies have focused on determining underlying effects of climate (temperature, humidity, rainfall) (Rose Vineer et al., 2016; Wang et al., 2014) and the development of different mechanistic models to predict temporal and spatial changes in parasite populations (Vineer et al., 2016). Using a climate simulation model, researchers were able to compare developmental success of *H. contortus* strains at varying temperatures (15-37°C) in real time to those predicted by the model. The researchers found that developmental success for three different *H. contortus* isolates was lower than that predicted by the climate simulation model, indicating the potential for overestimation of disease transmission risk at higher temperatures that are predicted in different models of climate change (Vineer et al., 2016). A different study was designed to explore the specific effects of moisture and humidity on ability of larvae to migrate out of fecal matter and onto pasture (Wang et al., 2014). The researchers found that light and regular rainfall allowed quicker emergence of larvae from a moist fecal pellet under humid conditions, but slower emergence of larvae from a fecal pellet in dry conditions (Wang et al., 2014). This indicates that humidity acts in combination with fecal moisture to increase larval migration (Wang et al., 2014). It was also determined that although larvae were not able to migrate out of dry fecal samples in the absence of rainfall, the larvae within the dry pellets survived, indicating the potential for fecal pellets to act as a reservoir in dry conditions (Wang et al., 2014). It has been further suggested that microclimate has

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a significant impact on fecal moisture content, which ultimately has an impact on larval migration, and may be more influential than macroclimate impacts (Wang et al., 2018).

6. Effect of Elevated Temperature and Humidity on Host Animal

6.1 The effect of heat stress on livestock production, immunity and health

Heat stress in sheep is associated with reduced feed intake, as well as changes in the metabolism of water, protein, energy, enzymatic reactions, and hormonal secretions (Marai et al., 2007). An increase in rectal temperature, respiration rate, as well as heart rate are typically used as indicators of heat stress in sheep. Another indicator of heat stress in sheep is thermal humidity index (THI). Exposure to THI above 80 results in elevated rectal temperatures and has shown to prevent lactating ewes from maintaining thermal balance, making it a good measure for determining heat stress (Sevi et al., 2001). Heat stress has been studied extensively as a factor negatively impacting milk production, performance and immunity in sheep, all of which are described in a review article by Sevi and Caroprese (2012). Exposure to heat (dry bulb temperature of 40°C) in combination with exercise has been shown to induce heat stress, leading to severe respiratory alkalosis (Bell et al., 1983). In a more recent study, Gaughan et al. (2016) found that heat stressed sheep showed an increase in respiration rate, rumen temperature, creatine kinase and creatinine as well as a decrease in alkaline phosphate (ALP), γ-glutamyl transpeptidase (GGT) and interleukin-1β (IL-1β) plasma concentrations compared to thermal neutral sheep, which indicates some impairment of the immune system.

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Heat stress has been studied extensively in swine and it has been recently determined that heat stress in combination with reduced feed intake alters intestinal integrity and increases endotoxin permeability within the GI tract (Pearce et al., 2013). Heat stress has also been examined as a factor influencing GI health in cattle and studies have shown that heat stressed cattle consume lower quantity of feed, causing a decrease in rumination (Aganga et al. 1990; Soriani et al. 2013). Moallem et al. (2010) further described the main negative impact caused by elevated THI to be a decrease in rumen temperature, leading to decrease in dry matter intake, and subsequently, lower milk yield. In sheep, heat stress (induced via exposure to elevated THI) leads to decreases in dry matter intake, rumen bacteria count, rumen osmolarity, rumen pH and digesta passage rates, among other GI parameters (Bernabucci et al., 2009). A decrease in feed intake can reduce the amount of heat that is generated by rumen digestion, which disrupts metabolism (Morand-Fehr and Doreau, 2001). Excessive panting is commonly observed as a response to heat stress in sheep, which causes nutritional requirements for maintenance to increase (Silanikove, 2000).

6.2 The effect of heat stress on sheep infected with Haemonchus contortus

It is known that plasma cortisol (indicator of stress in livestock) levels are influenced by environmental temperatures (Nejad et al., 2014). Swarnkar and Singh (2017) looked at differences between sheep bred for resistance to *H. contortus* infection and susceptible sheep in relation to heat stress. They found that both groups remained in stress during the dry/hot season regardless of resistance, but the susceptible sheep showed signs for higher stress (high cortisol levels and fecal egg count) for a longer period of time moving from the dry/hot season into the humid/hot season. These

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differences indicate that resistant animals are better able to withstand stress in the presence of infection better than those animals who are susceptible and that seasonal variation has a direct impact on the stress response of *H. contortus* infected sheep. In a more recent study performed in the same region (Avikanager, Rajashan), researchers found that THI (thermal humidity index) showed a positive relationship with both fecal egg count and abomasal worm burden (Swarnkar and Singh, 2018). The researchers found a positive correlation between FEC and THI during the period of stress (April-June) and again in the period of non-stress (October to December), indicating resumption of development in hypobiotic larvae. During the period of April-June, this could be due to unfavorable, stressful conditions for the host, while during the period of October-December, this may be caused by induction of hypobiosis due to non-stressful status of the host.

7. Age of *Haemonchus contortus* larvae

7.1 Effect of Age of H. contortus on Exsheathment

Castaneda-Ramirez et al. (2017) examined the direct effects of age of stored *H. contortus* larvae on exsheathment inhibition using *Acacia pennatula* extracts (Castaneda-Ramirez et al., 2017). They found that at a concentration of 400 µg/mL, exsheathment inhibition decreased significantly as the age of the larvae increased from 1 to 7 weeks. While exsheathment inhibition at 400 µg/mL *Acacia* extract was 100% for L3 aged 1-4 weeks old, exsheathment inhibition dropped to 39.5% and did not exceed 77.7% in weeks 5-7 (Castaneda-Ramirez et al., 2017). Furthermore, 1200 µg/mL *Acacia pennatula* extract was needed to achieve 100% exsheathment inhibition in 5-week-old L3 larvae and a concentration beyond 1200 µg/mL would be needed to

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achieve 100% exsheathment inhibition in 6-7-week-old L3 larvae (Castaneda-Ramirez et al., 2017). The study also looked at the effect of larva age on larval migration and motility, finding that both decreased as age increased (Castaneda-Ramirez et al., 2017). Older L3 larvae may exsheath more readily than younger L3 larvae due to lower energy reserves and the accomplishment of various physiological ‘steps’ needed for exsheathment to occur (Hertzberg et al., 2002; Castaneda-Ramirez et al., 2017). Future research should investigate morphological changes caused by *Acacia pennatula* in L3 larvae of different ages, as well as evaluating effects of age using different isolate strains of *H. contortus*. At the present time, this is the only study that looks at the direct correlation between age of *H. contortus* larvae and exsheathment. No studies to date have examined the age of the adult worm (rather than the age of the larva) in an infection and how this may impact larval exsheathment.

8. Summary and Conclusion

Gastrointestinal nematode (GIN) infection poses a major threat to the health of small ruminants, negatively impacting economic viability of producers. Life cycle stages including egg hatch and larval exsheathment are commonly studied in GIN anthelmintic research. Donor animals are commonly infected to produce eggs and larvae to be used in various *in vitro* and *in vivo* assays, on a year-round basis. Variability observed in previous studies has suggested that multiple factors may influence larval performance in *in vitro* and *in vivo assays*. While previous research has explored environmental factors influencing the life cycle of *H. contortus,* there is very limited work that examines the direct relationship between seasonal fluctuations and egg and larvae performance in *in vitro* and *in vivo* egg hatch and exsheathment assays.

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There is also a lack of knowledge of how the age of the adult worm in a host infection can influence performance of their offspring (larvae) in different assays. Studying the effects of season and age of infection on performance of larvae and eggs in various assays can ultimately improve the quality of future research and shed light on 'best practices' when using donor animals.

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CHAPTER II

The effect of season on the life cycle of *Haemonchus contortus* in experimentally infected lambs

To be submitted to Veterinary Parasitology

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The effect of season on the life cycle of *Haemonchus contortus* in experimentally infected lambs.

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Abstract

Haemonchus contortus eggs and larvae are routinely used in studies of anthelmintic compounds to combat gastrointestinal nematode infection. Although it is known that *Haemonchus contortus* larvae arrest their development within the host during unfavorable environmental conditions, it is unknown how time or year or age of the adult worm affects the hatchability of eggs or the efficiency with which the larvae exsheath artificially *in vitro* or *in vivo* within the rumen. The main objective of this study was to determine the role of time of year and age of adult *H. contortus* on hatchability of eggs and ability of resulting infective larvae to exsheath artificially *in vitro* or *in vivo*. A secondary objective of the study was to asses correlation between *in vitro* and *in vivo* assays. Two lambs were experimentally infected (10,000 L3) at the start of each season (autumn equinox 1, Winter solstice, vernal equinox, summer solstice, autumn equinox 2; n=2/season). Donor lamb feces were collected monthly for up to six months following each infection. Egg hatchability was tested *in vitro* and L3 were subjected to *in vitro* exsheathment using artificial CO_2 and *in vivo* exsheathment within ruminally fistulated ewes. There was no effect of worm age ($p = 0.4$), season ($p = 0.09$), or worm age*season ($p = 0.07$) on egg hatchability. There was a worm age*season effect on both *in vitro* ($p \leq 0.0002$) and *in vivo* ($p \leq 0.0002$) viability as well as *in vitro* ($p \leq 0.0001$) and *in vivo* ($p \leq 0.0077$) exsheathment. The *in vivo* assay yielded more variable viability across seasons compared to *in vitro*, but for both, viability of larvae from worms aged one month was lower during the Fall 1 and Winter cycles compared to all other cycles. The *in vitro* assay yielded more variable exsheathment across and within seasons compared to the *in vivo* assay, especially

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during the Fall 1, Summer and Fall 2 cycles. When comparing the *in vitro* to the *in vivo* assay, *in vitro* yielded higher viability while *in vivo* yielded higher exsheathment across various months and seasons of testing ($p \leq 0.05$). The results of this study can help to identify constraints posed by seasonal changes in using *H. contortus* L3 in *in vitro* and *in vivo* assays, as well as highlight the importance of age of an infection producing the L3.

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1. Introduction

Gastrointestinal nematode (GIN) infections (particularly Haemonchus contortus infection) pose a major threat to the health of small ruminants and have been identified as a major limitation for pasture-based production of sheep and goats (Hoste et al., 2006; Veríssimo et al., 2012). *Haemonchus contortus* life cycle stages, such as the egg hatch and larval exsheathment are commonly studied in anthelmintic research. These life cycle stages are typically examined using experimentally infected sheep and goats on a year-round basis to provide fecal eggs and larvae used in *in-vitro* and *in-vivo* assays (Brunet et al., 2007; Alonso-Diaz et al., 2008; von Son-de Fernex et al., 2012; Araújo et al., 2017; Assis et al. 2003; Marie-Magdeleine et al. 2009; Barone et al., 2018).

Multiple assays have been developed to assess exsheathment *in vitro* (Bahuaud et al., 2006; Alonso-Diaz et al., 2008; Son-de Fernex et al., 2012; Araújo et al., 2017; Conder and Johnson, 1996; Barone et al. 2018). The sodium hypochlorite method (Bahuaud et al., 2006) is more commonly utilized (Alonso-Diaz et al., 2008; Son-de Fernex et al., 2012; Araújo et al., 2017), however it has been suggested that the CO_2 method improves viability and infectivity (Conder and Johnson, 1996). A considerable amount of variability has been observed across *in vivo* studies in relation to methodology, as well as time for exsheathment to occur (Sommerville et al., 1957; Hertzberg et al., 2002; Brunet et al., 2007). While results from previous *in vivo* studies have been observationally compared to results using different *in vitro* methods--sodium hypochlorite method (Bahuaud et al., 2006; Brunet et al., 2007) and rumen simulation (RUSITEC) technique (Czerkawski & Breckenridge, 1977; Hertzberg et

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al., 2002)-- they have not been compared statistically to one another, and the CO_2 method (Condor and Johnson, 1996) has yet to be directly compared to *in vivo* methods.

The effect of varying laboratory incubation temperatures on *H. contortus* eggs and L3 larvae has been previously studied (Silverman and Podger, 1964; Slocombe and Whitlock, 1970; Smith-Buijs and Borgsteede, 1986; Jasmer et al., 1986; Mckenna et al., 1998; Bekelaar et al., 2018). Prolonged exposure to controlled cold temperatures (<10°C) can negatively impact *H. contortus* egg hatchability, the development of eggs to third stage larvae and viability of third stage larvae (Smith-Buijs and Borgsteede, 1986; Jasmer et al., 1986; Mckenna et al., 1998; Chylinski et al., 2015; Ilieve et al., 2018). The effect of elevated laboratory incubation temperature on L3 larvae has also been studied, showing a negative correlation between viability and increased temperature ≥ 40°C (Ilieve et al., 2018) and it has been further determined that heat shock (rapid change in temperature to 40°C) may be an important factor in inducing exsheathment (Bekelaar et al., 2018; Bekelaar et al., 2019).

Haemonchus contortus larvae transition into a hypobiotic state, to increase survival during adverse environmental conditions (Gatongi et al., 1998;). Hypobiosis of *H. contortus* has been studied extensively in different areas of the world in both temperate and tropical climates (Fakae, 1990; Connan, 1971; McKenna, 1974; Capitini et al., 1990; Uriate et al., 2003; Waller et al., 2004; Sargison et al., 2007; Hosseini et al., 2012). A considerable amount of variability has been observed across various studies examining hypobiosis patterns in temperate climates (Capitini et al., 1990; Connan, 1975; Blitz and Gibbs, 1972; Eysker, 1981; McKenna, 1973). The

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source of L3 larvae (culture and storage conditions) used for study subject infection in different studies has been identified as a potential factor influencing variability in observed hypobiosis patterns (Capitini et al., 1990). Other climatic factors have been studied in relation to *H. contortus* development, such as humidity which has been found to act in combination with fecal moisture to increase larval migration, which is ultimately influenced by microclimate as opposed to macroclimate (Wang et al., 2014; Wang et al., 2018). Studies exploring hypobiosis and climate factors have focused on impacts related to larval development and migration patterns, but very limited work exists examining the impact of season specifically on larval exsheathment both *in vitro* and *in vivo*.

It is common practice to store experimentally obtained L3 larvae in a refrigerator (4-8°C), with most published studies noting that larvae used in their studies had been in storage for no longer than three months (Hertzberg et al., 2002; Brunet et al., 2007). Previous work has been conducted to explore the effect of storage age of L3 (time in storage) as a factor in the *in vitro* assessment of anthelmintic properties of tannin containing plant extracts (Castañeda-Ramírez et al., 2017). Increasing age of stored L3 has a negative impact on larval motility and migration assays, as well as exsheathment inhibition (Castañeda-Ramírez et al., 2017). The impact of adult worm age on L3 larvae exsheathment has not been studied.

The variability in previous work in addition to our own findings supports a need for research exploring potential environmental factors (seasonal fluctuations) influencing larval development. There is a lack of concrete data related to the impact of seasonal changes, as well as the impact of age of the adult worm itself on egg and

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larval performance in *in vitro* and *in vivo* assays. The first objective of this study was to determine if age of the adult worms in the host animal affects the ability of their eggs to hatch as well as viability and exsheathment of the resulting third stage *H. contortus* larvae used in *in vitro* and *in vivo* assays. The second objective of this study was to determine the effect of season on the adult worms in the host animal and on the ability of the eggs from these worms to hatch *in vitro* and larvae to exsheath *in vitro* and *in vivo*. The third and final objective of this study was to compare results for larval viability and exsheathment obtained from the *in vitro* and *in vivo* assay in order to determine correlation between the two assays.

2. Materials and Methods

2.1 Study Design

The study design is depicted in Figure 1. The project was designed to run in a series of four cycles corresponding to the beginning of the four seasons of the year 1) Fall (autumnal equinox, September 22, 2017); 2) Winter (Winter solstice, December 21, 2017); 3) Spring (vernal equinox, March 20, 2018); 4) Summer (summer solstice, June 21, 2018). A fifth cycle was added as a replicate of the original fall cycle, which is termed 'Fall 2' (autumnal equinox, September 22, 2018). Daily temperature, humidity and precipitation data was obtained from the weather station at the University of Rhode Island's Peckham Farm. Each cycle began (time (t) = 0) with the experimental infection of two donor lambs with 10,000 *H. contortus* L3 larvae obtained from previously infected donor animals. The larvae obtained from the previously donors was between 1 and 3 weeks old (kept in storage at 4°C for no longer

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than 3 weeks prior to infection dates). All donor animals were raised indoors from birth, with the exception of the Fall 2 lambs, which had limited access to pasture prior to being dewormed for infection. Fecal egg counts (FEC) and packed cell volume (PCV) were conducted at t = 0 and every week thereafter through all weeks of infection. Fecal samples were collected from each lamb for both egg recovery and larval culture beginning at four weeks of infection and continuing every four weeks through 16-20 weeks of infection (sampling was discontinued when egg counts fell below 300 eggs per gram). Upon the collection of the fecal sample, eggs were extracted from the feces and used in the egg hatch assay to determine egg hatchability. The remainder of the fecal sample was used to prepare fecal cultures that yielded L3 larvae. The larvae were extracted from the culture samples and used in an *in vitro* exsheathment assay using CO_2 treatment, as well as in an *in vivo* exsheathment assay using 4 ruminally fistulated ewes.

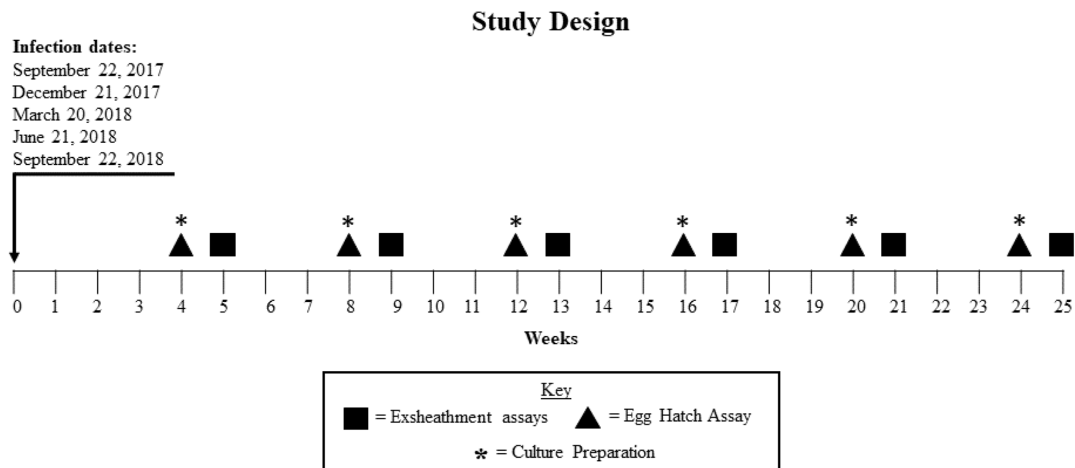

Figure 1. Study Design

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2.2 Study Subjects & Infection Monitoring

All procedures used in this study were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Rhode Island. Two sets of twin Dorset ram lambs born in March of 2017 were used for cycles Fall 1 and Winter (one set of twins per cycle). One set of twin Dorset-cross ewe lambs, born in September of 2017, was used for the Spring cycle and one set of genetically related (same sire) Dorset-cross ewe lambs, also born in September of 2017, were used for the Summer cycle. Two genetically related (same sire) Dorset ram lambs, born in April of 2018, were used for the Fall 2 cycle. All lambs used for each seasonal cycle were experimentally infected with 10,000 *H. contortus* (2 lambs per season) at the start of cycle. Donor lambs for the Fall 1, Winter, Spring and Summer cycles were raised indoors and had no previous exposure to nematode infection. The donor lambs used in the Fall 2 cycle were exposed to pasture conditions for one month (August 2018) and were dewormed prior to being infected for the trial. Four fistulated Dorset-cross ewes (~ 5 years old), housed at Peckham Farm, were used for *in vivo* exsheathment assays. Fistulated ewes were housed indoors with no pasture access. All animals (donor and fistulated) were fed a diet of grass mix hay produced at Peckham Farm, as well as a 16% complete commercial sheep pellet to meet their daily nutritional requirements.

2.2.1 Packed Cell Volume and Fecal Egg Count

Blood samples were taken weekly from each lamb and processed to determine packed cell volume (PCV) using the micro-hematocrit centrifuge method. Weekly

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fecal egg counts (FEC) were determined on all infected animals using the modified McMaster technique (Whitlock, 1948) and a commercially available sodium nitrate flotation solution (Fecasol®, Vetoquinol U.S.A., Inc., Fort Worth, TX, USA, specific gravity- 1.200) with each egg counted representing 50 eggs/gram of feces.

2.3 Egg Recovery and Egg Hatch Assay

Fecal samples were collected rectally from donor lambs to be used for egg recovery. The feces collected were placed in a cooler and briefly (< 2 hours) stored in the refrigerator to avoid premature hatching of eggs. The fecal samples were then combined with water to create a slurry mixture, which was poured over sieves of decreasing sizes (1 mm, 355, 150, 38 and 25 µm) (Assis et al., 2003; Marie-Magdeleine et al., 2009; Barone et al., 2018). Eggs were collected from the 38 and 25 µm sieves using a salt solution (Fecasol®, Vetoquinol U.S.A., Inc., Fort Worth, TX, USA). The egg-containing solution was centrifuged, and eggs were collected using cover slips, rinsed using water and the final aqueous solution of eggs was used in the egg hatch assay that day. The egg hatch assay was conducted using established procedures (Assis et al. 2003; Marie-Magdeleine et al. 2009). Eggs were added (100 eggs in 100 µl of water per well) to a 24-well flat-bottomed microtiter plate (Corning™, Falcon™, Polystyrene Microplates, Corning Life Sciences, Tewksbury, MA, USA). Fecal water and DMSO (dimethyl sulfoxide) were added (1,900 µl) to each well to bring the final volume to 2 ml (Barone et al., 2018). A set of 5 wells was run for each lamb. The wells were incubated at 26°C for 24 hours and read under a microscope the following day to quantify eggs and larvae in order to determine percent egg hatchability (% Hatchability= # larvae/# eggs + larvae).

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2.4 Larval Recovery

Fecal samples were collected from infected donor lambs via rectal palpation to prepare fecal cultures using a modified version of the Baermann technique (Zajac and Conboy, 2012). The fecal samples collected were placed in a refrigerator for brief (< 2 hours) storage before the cultures were prepared. For cultures, fecal samples were maintained in a humid environment and incubated at approximately 25°C for 7-14 days. The fecal samples rested on cheesecloth and were flooded with water to recover hatched larvae. The flooded cultures sat for a minimum of 8 hours at room temperature in order for larvae to settle within the water solution. Excess water was siphoned off the top so that a concentrated larval solution was obtained. The concentrated larval solution was prepared in order to be used in the *in vitro* and in *vivo* exsheathment assays.

2.5 Exsheathment Assays

2.5.1 In vitro CO_2 Exsheathment Assay

The concentrated larval solution obtained from cultures was transferred to the laboratory prior to the set up of the *in vitro* CO_2 exsheathment assay. The *in vitro* CO_2 exsheathment assay was conducted according to previously published procedures (Condor and Johnson, 1996; Barone et al., 2018). Briefly, 2,000 L3 larvae were added to a polypropylene tube and Earle's Balanced Salt Solution (EBSS, Sigma-Aldrich®, Inc., Natick, MA, USA) was added to bring the volume to 1 mL. An additional 1 mL of water was added to the tube to bring the final volume to 2 mL. Three experimental tubes and one 'no CO_2' control tube was prepared for each of the two fecal samples

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(four duplicates for each lamb). The three experimental tubes were exposed to CO_2 treatment using a modified version of the technique proposed by Conder and Johnson (1996). The four tubes were covered using Parafilm M® (Parafilm M®, Bemis Company, Inc., Neenah, WI). A glass pipet tip, connected to the CO_2 tank was pushed through the Parafilm M® covering so that it was suspended in the larval solution. Solutions were bubbled with CO_2 for 15 minutes each and tubes were resealed using Parafilm M® before being capped (Barone et al., 2018). The tubes were bubbled at temperatures slightly below average room temperature (18-22 °C). The capped tubes were incubated at 37°C for 18 hours. After the incubation period, the larvae were observed for viability (motility observed for 5 seconds) and presence of sheath. At least 100 live larvae were counted for each tube and results were expressed as percent viability (including exsheathed and ensheathed) and percent live exsheathment (percent motile exsheathed). Percentages for both viability and exsheathment were adjusted to the negative control ('No CO_2) values for each individual assay. Adjustments were made using the following formula: live exsheathment or viability (%) = 100 - (A – B)/(A) x 100 (Acharya et al., 2014). Where A = % viable or exsheathed in the water control and B = % viable or exsheathed in the CO_2 replicates.

2.5.2 In vivo Exsheathment Assay

The L3 larvae were maintained at 4-6 °C for less than 24 hours followed by an 18-24 hour readjustment period to room temperature prior to use in the *in vivo* assay. The exsheathment assay used for this experiment was modified from that of Brunet et al. (2007). Both concentration and viability were determined for each set of larvae. A total of 2,000 ensheathed L3 larvae were added to a capsule composed of a piece

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Tygon® tubing (ID 9.5, OD 14.3 mm, Fisher Scientific, Hampton, NH) and two 8 µm Nunc ™ Cell Culture Inserts (#140629, Thermo Scientific, Waltham, MA) on each end to allow free flow of rumen fluid throughout the tube. A total of 8 capsules were prepared—4 capsules per donor lamb (1 capsule per donor per fistulated ewe). The capsules were each placed in a heat-sealed concentrate bag (R510, ANKOM Technology, Macedon, NY), to prevent large particles in the rumen from clogging the insert membrane. The enveloped capsules were tied to a 20 cm cord and suspended in the rumen of a fistulated ewe (two capsules/ewe). Four ewes were used, each receiving one capsule from each lamb. The capsules were removed after an exposure period of 8 hours. The larvae in the capsules were observed for percent viability (motility observed for 5 seconds) and presence of sheath with approximately 150 live larvae being counted from each capsule. Results were expressed as percent viability (including ensheathed and exsheathed L3) and percent live exsheathment (percent motile exsheathed L3).

2.6 Statistical Analysis

2.6.1 Egg Hatch, in vitro and in vivo exsheathment assays

Data were analyzed using the GLIMMIX procedure in SAS (SAS Institute Inc., Cary, NC). The model included terms cycle (representing each seasonal cycle); worm age (age of donor infection); and cycle*worm age. An adjustment for multiple comparisons test was performed using Tukey-Kramer for simple effect comparisons of cycle*worm age least square means by cycle and by age. Significance of least square means was defined as $p < 0.05$.

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2.6.2 Comparison of in vitro to in vivo assay

Data was analyzed using an analysis of variance (ANOVA) and means were separated with Dunnett's t-test using the GLM procedure in SAS.

3. Results:

3.1 Experimental Infections

The amount of viability and live exsheathment data (*in vitro* and *in vivo*) that were collected throughout the trial varied between seasons due to persistence of the experimental infections. Donor infections persisted for at least 4 months for all 5 seasons. The Fall 1 infection lasted 4 months, Winter and Summer lasted 5 months and Spring and Fall 2 infections persisted through 6 months, however the larvae harvested for the Fall 2 cycle at 6 months did not yield enough larvae for both *in vitro* and *in vivo* assays to be performed, so only the *in vitro* assay was performed.

3.2 Larval Cultures

Each month, L3 needed for *in vitro* and *in vivo* assay was cultured from donor lamb fecal samples. Unexpectedly, the time it took for larvae in culture to reach the L3 stage varied between season and between twin donor animals within the same season (Table 1).

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Table 1. Variability in rate of L3 development in culture

Worm Age → (Months) **Season ↓**	1	2	3	4	5	6
	Time in Culture (Days)					
Fall 1	7	7	7	7	--	--
Winter	7	7	7	9 [a]	9 [a]	--
Spring	9 [a]	9 [a]	14 [b]	14	14	14
Summer	14 [b]	14	14	14	14	--
Fall 2	14 [b]	14	14	14	14	14

[a] Larvae for one donor failed to develop to L3 stage at 7 days, but developed to L3 at 9 days. A 9-day culture period was used.
[b] Larvae for one donor failed to develop to L3 stage at both 7 and 9 days, but developed to L3 at 14 days. A 14-day culture period was used for the duration of the cycle due to continued failures at both 7 and 9 days for all consecutive months for one donor.
-- No sample; infection did not persist in donor animals.

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3.3 Effect of worm age and season on percent egg hatchability

Hatchability across all cycles throughout the entire trial (data not shown) was ≥ 90% and ranged from 91 - 99%. Average percent hatchability across all months of testing for each season was lowest for the Summer cycle (95%) and highest for the Winter cycle (98%). Average percent hatchability across all seasons of testing for each month ranged from 96 - 97%. There were no statistically significant differences for effect of worm age ($p = 0.4$), effect of season ($p = 0.09$), or effect of worm age*season ($p = 0.07$).

3.4 Effect of worm age and season on in vitro and in vivo percent viability

3.4.1 Effect of season on in vitro and in vivo percent viability

Viability data for both the *in vitro* and *in vivo* methods were averaged across lambs and replicates for each month of testing for each seasonal cycle (Table 2). *In vitro* ($p \leq 0.0002$) and *in vivo* ($p \leq 0.0001$) viability varied by worm age across seasons. *In vitro* viability of larvae from eggs collected one month post-infection was higher during the Summer and Fall 2 cycles than the other cycles and larval viability from one-month old worms during the Spring cycle was higher than the Winter cycle ($p \leq 0.05$) (Table 2). Season had no effect on viability for larvae from eggs collected > one month post infection.

In vivo larval viability was lower during the Fall 1 and Winter cycles for larvae from one-month old worms and lower during the Fall 1 cycle only for larvae from two and four-month-old worms ($p \leq 0.05$) (Table 1). *In vivo* larval viability was lower during the Winter cycle for five-month-old worms than the subsequent seasons (Table

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2). There was no effect of cycle for larval viability for three-month-old worms, with all larval viability >96% for all seasons ($p \leq 0.05$) (Table 2). Spring was the only seasonal cycle that yielded *in vivo* larval viability data for six-month-old worms.

3.4.2 Effect of worm age on in vitro and in vivo percent viability

Viability data for both the *in vitro* and *in vivo* methods was averaged for each month of testing for each seasonal cycle (Table 3). *In vitro* ($p \leq 0.0002$) and *in vivo* ($p \leq 0.0001$) viability varied across different worm ages. *In vitro* viability of larvae from worms aged 1 month were lower than the viability of larvae from older worms within the Fall 1 and Winter cycles and larval viability for larvae from one-month old worms was lower than the larval viability from worms aged 5 and 6 months within the Spring cycle ($p \leq 0.05$) (Table 3). There were no differences in larval viability across worm ages within the Summer and Fall 2 cycles ($p \leq 0.05$) (Table 3).

Within the Fall 1 cycle the *in vivo* viability of larvae from one-month old worms was lower than older worms and the larval viability of the two and four-month-old worms was lower than the larval viability from the three-month-old worms ($p \leq 0.05$) (Table 3). During the Winter cycle, the larval viability from the one-month old worms was again lower than larvae from the older worms and the larval viability from the five-month-old worms was less than that of the two and three-month-old worms ($p \leq 0.05$) (Table 3). There were no differences in viability between any worm ages during the Spring, Summer or Fall 2 cycle (Table 3).

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Table 2. Effect of season on percent viability (*in vitro* and *in vivo*) of *Haemonchus contortus* larvae (L3) offspring from worms aged 1- 6 months.

Season → **Worm Age** **(Months) ↓**	Fall 1	Winter	Spring	Summer	Fall 2
In vitro [1]			**Percent Viability (%)**		
1	89 ± 2 [ab]	90 ± 1 [a]	95 ± 1 [b]	100 ± 1 [c]	100 ± 1 [c]
2	97 ± 1	96. ± 1	100 ± 2	99 ± 1	99 ± 1
3	98 ± 1	99 ± 2	100 ± 2	100 ± 1	100 ± 1
4	97 ± 1	99. ± 1	99 ± 1	100 ± 1	99 ± 1
5	--	100 ± 1	99 ± 1	100 ± 1	100 ± 1
6	--	--	99 ± 1	--	100 ± 2
In vivo [2]					
1	68 ± 3 [a]	70 ± 2 [a]	92 ± 2 [b]	98 ± 2 [b]	99 ± 2 [b]
2	81 ± 2 [a]	97 ± 2 [b]	90 ± 3 [b]	97 ± 2 [b]	98 ± 2 [b]
3	96 ± 2	97 ± 3	99 ± 3	98 ± 2	99 ± 2
4	85 ± 2 [a]	94 ± 2 [b]	98 ± 2 [b]	98 ± 2 [b]	99 ± 2 [b]
5	--	88 ± 2 [a]	98 ± 2 [b]	97 ± 2 [b]	99 ± 2 [b]
6	--	--	98 ± 2	--	--

[1]Larvae (2,000 ensheathed L3/replicate x 3 replicates per donor lamb(N=2)) were exposed to CO_2 bubbling to induce exsheathment and incubated at 37°C for 18 hours. After incubation, all larvae were determined to be alive or dead (% live viability = total live L3/total L3).
[2]Larvae (2,000 ensheathed L3/replicate x4 per donor lamb (N=2)) were added to a tygon tube capsule and suspended in the rumen of fistulated ewes for an 8-hour period. After rumen exposure, all larvae were determined to be alive or dead (% live viability = total live L3/total L3).
All values are least square mean ± SEM
-- No sample; infection did not persist in donor animals.
Means with different superscripts across rows differ significantly ($p \leq 0.05$)

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Table 3. Effect of worm age on percent viability (*in vitro* and *in vivo*) of *Haemonchus contortus* larvae (L3) offspring within seasons.

Worm Age → **(Months)** **Season ↓**	1	2	3	4	5	6
In vitro [1]	**Percent Viability (%)**					
Fall 1	89 ± 2 [a]	97 ± 1 [b]	98 ± 1 [b]	97 ± 1 [b]	--	--
Winter	90 ± 1 [a]	96 ± 1 [b]	99 ± 2 [b]	99 ± 1 [b]	100 ± 1 [b]	--
Spring	95 ± 1 [a]	100 ± 2 [ab]	100 ± 2 [ab]	99 ± 1 [ab]	99 ± 1 [b]	99 ± 1 [b]
Summer	100 ± 1	99 ± 1	100 ± 1	100 ± 1	100 ± 1	-
Fall 2	100 ± 1	99 ± 1	100 ± 1	99 ± 1	100 ± 1	100 ± 2
In vivo [2]						
Fall 1	68 ± 3 [a]	81 ± 2 [b]	96 ± 2 [c]	85 ± 2 [b]	--	--
Winter	70 ± 2 [a]	97 ± 2 [b]	97 ± 3 [b]	94 ± 2 [bc]	88 ± 2 [c]	--
Spring	92 ± 2	90 ± 3	99 ± 3	98 ± 2	98 ± 2	98 ± 2
Summer	98 ± 2	97 ± 2	98 ± 2	98 ± 2	97 ± 2	--
Fall 2	99 ± 2	98 ± 2	99 ± 2	99 ± 2	99 ± 2	--

[1]Larvae (2,000 ensheathed L3/replicate x 3 replicates per donor lamb(N=2)) were exposed to CO_2 bubbling to induce exsheathment and incubated at 37°C for 18 hours. After incubation, all larvae were determined to be alive or dead (% live viability = total live L3/total L3).
[2]Larvae (2,000 ensheathed L3/replicate x4 per donor lamb (N=2)) were added to a tygon tube capsule and suspended in the rumen of fistulated ewes for an 8-hour period. After rumen exposure, all larvae were determined to be alive or dead (% live viability = total live L3/total L3).
All values are least square mean ± SEM
-- No sample; infection did not persist in donor animals.
Means with different superscripts across rows differ significantly ($p \leq 0.05$)

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3.5 Effect of worm age and season on in vitro and in vivo percent live exsheathment

3.5.1 Effect of season on in vitro and in vivo % live exsheathment

Exsheathment data for both the *in vitro* and *in vivo* methods was averaged for each month of testing for each seasonal cycle (Table 4). *In vitro* ($p \leq 0.0001$) and *in vivo* ($p \leq 0.0077$) exsheathment varied within worm age across season. When comparing *in vitro* larval exsheathment of one-month old worms across season, the Fall 2 cycle was dramatically lower than all other seasons, and the larval exsheathment during the Summer cycle was greater than that observed in the Winter and Spring cycle. Significantly depressed exsheathment of larvae from two-month-old worms was observed in both Fall cycles as compared to the other seasons and additionally the exsheathment during the Summer cycle was also lower than that measured during the Winter cycle. For three-month-old worms the Summer exsheathment percentage was depressed over that of all other seasons. Larval exsheathment from four-month-old worms was also depressed during the Summer cycle but also during the Fall 1 cycle. Finally, the exsheathment of larvae from five-month-old worms was dramatically lower during the Summer cycle than all other cycles and the exsheathment during the Fall 2 cycle was significantly greater than all other cycles. ($p \leq 0.05$) (Table 4).

In contrast to the variability observed for the *in vitro* larval exsheathment, there were no differences across seasonal cycles for *in vivo* exsheathment of larvae from one, two and four-month-old worms. The *in vivo* exsheathment of larvae from three-month-old lambs was greater during the Fall 1 cycle than for the Winter, Summer and Fall 2 cycles. There was a suppression in the exsheathment of larvae from five-

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month-old worms during the Summer cycle as compared to the Winter cycle ($p \leq 0.05$) (Table 4).

3.5.2 Effect of worm age on in vitro and in vivo percent live exsheathment

Live exsheathment data for both the *in vitro* and *in vivo* methods was averaged for each month of testing for each seasonal cycle (Table 5). *In vitro* ($p \leq 0.0001$) and *in vivo* ($p \leq 0.0077$) exsheathment varied within season across different worm ages. *In vitro* exsheathment for the Fall 1 cycle was highly variable across the different worm ages with a significant depression in exsheathment of larvae from two and four-month-old worms. Larval exsheathment did not vary across worm ages during the Winter cycle. During the Spring cycle the exsheathment of larvae from one and six-month-old worms differed. Exsheathment of larvae during the Summer and Fall 2 cycles was highly variable across the various worm ages with the highest larval exsheathment observed from one-month old worms during the Summer cycle and from five-month-old worms during the Fall 2 cycle.

In vivo exsheathment during the Fall 1 cycle was lower for larvae from one and two-month-old worms, as compared to three-month-old worms ($p \leq 0.05$) (Table 5). No differences were observed in larval exsheathment from worms of any age during the Winter and Spring cycles. During the Summer cycle, exsheathment of larvae from four-month-old worms was greater than that from five-month-old worms and during the Fall 2 cycle exsheathment percentage was greater for the larvae from four-month-old worms than the one-month old worms ($p \leq 0.05$) (Table 5).

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Table 4. Effect of season on percent exsheathment (*in vitro* and *in vivo*) of *Haemonchus contortus* larvae (L3) offspring from worms aged 1- 6 months.

Season → **Worm Age** **(Months) ↓**	Fall 1	Winter	Spring	Summer	Fall 2
In vitro [1]	**Percent Exsheathment (%)**				
1	64 ± 6^{ab}	63 ± 3^{a}	68 ± 3^{a}	81 ± 3^{b}	21 ± 3^{c}
2	24 ± 4^{c}	67 ± 3^{a}	67 ± 5^{ab}	51 ± 3^{b}	31 ± 3^{c}
3	68 ± 4^{a}	69 ± 5^{a}	67 ± 5^{a}	24 ± 3^{b}	77 ± 3^{a}
4	22 ± 3^{a}	63 ± 3^{b}	56 ± 6^{b}	15 ± 3^{a}	60 ± 3^{b}
5	--	68 ± 3^{a}	62 ± 3^{a}	17 ± 3^{b}	97 ± 3^{c}
6	--	--	51 ± 3	--	45 ± 5
In vivo [2]					
1	63 ± 8	70 ± 6	64 ± 6	51 ± 6	49 ± 6
2	69 ± 6	74 ± 6	77 ± 8	72 ± 6	64 ± 6
3	90 ± 6^{a}	63 ± 8^{b}	75 ± 8^{ab}	61 ± 6^{b}	62 ± 6^{b}
4	71 ± 6	74 ± 6	56 ± 6	72 ± 6	77 ± 6
5	--	78 ± 6^{a}	64 ± 6^{ab}	50 ± 6^{b}	61 ± 6^{ab}
6	--	--	74 ± 6	--	--

[1]Larvae (2,000 ensheathed L3/replicate x 3 replicates per donor lamb(N=2)) were exposed to CO_2 bubbling to induce exsheathment and incubated at 37°C for 18 hours. After incubation, all larvae were determined to be alive or dead (% live exsheathment = total live exsheathed L3/total live L3).
[2]Larvae (2,000 ensheathed L3/replicate x4 per donor lamb (N=2)) were added to a tygon tube capsule and suspended in the rumen of fistulated ewes for an 8-hour period. After rumen exposure, all larvae were determined to be alive or dead (% live exsheathment = total live exsheathed L3/total live L3).
All values are least square mean ± SEM
-- No sample; infection did not persist in donor animals.
Means with different superscripts across rows differ significantly ($p \leq 0.05$)

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Table 5. Effect of worm age on percent exsheathment (*in vitro* and *in vivo*) of *Haemonchus contortus* larvae (L3) offspring within seasons.

Worm Age → **(Months)** **Season ↓**	1	2	3	4	5	6
In vitro [1]			**Percent Exsheathment (%)**			
Fall 1	64 ± 6 [a]	24 ± 4 [b]	68 ± 4 [a]	22± 3 [b]	-	-
Winter	63 ± 3	67 ± 3	69 ± 5	63 ± 3	68 ± 3	-
Spring	68 ± 3 [a]	67 ± 5 [ab]	67 ± 5 [ab]	56 ± 6 [ab]	62 ± 3 [ab]	51 ± 3 [b]
Summer	81 ± 3 [a]	51 ± 3 [b]	24 ± 3 [c]	15 ± 3 [c]	17 ± 3 [c]	-
Fall 2	21 ± 3 [a]	31 ± 3 [ab]	77 ± 3 [d]	60 ± 3 [c]	97 ± 3 [e]	45 ± 5 [bc]
In vivo [2]						
Fall 1	63 ± 8 [a]	69 ± 6 [a]	90 ± 6 [b]	71 ± 6 [ab]	-	-
Winter	70 ± 6	74 ± 6	63 ± 8	74 ± 6	78 ± 6	-
Spring	64 ± 6	77 ± 8	75 ± 8	56 ± 3	64 ± 6	74 ± 6
Summer	51 ± 6 [ab]	72 ± 6 [ab]	61 ± 6 [ab]	72 ± 6 [a]	50 ± 6 [b]	-
Fall 2	49 ± 6 [a]	64 ± 6 [ab]	62 ± 6 [ab]	77 ± 6 [b]	61 ± 6 [ab]	-

[1]Larvae (2,000 ensheathed L3/replicate x 3 replicates per donor lamb(N=2)) were exposed to CO_2 bubbling to induce exsheathment and incubated at 37°C for 18 hours. After incubation, all larvae were determined to be alive or dead (% live exsheathment = total live exsheathed L3/total live L3).
[2]Larvae (2,000 ensheathed L3/replicate x4 per donor lamb (N=2)) were added to a tygon tube capsule and suspended in the rumen of fistulated ewes for an 8-hour period. After rumen exposure, all larvae were determined to be alive or dead (% live exsheathment = total live exsheathed L3/total live L3).
All values are least square mean ± SEM
-- No sample; infection did not persist in donor animals.
Means with different superscripts across rows differ significantly ($p \leq 0.05$)

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3.6 Comparing the in vitro assay to in vivo assay

3.6.1 Comparing in vitro vs. in vivo percent viability

Viability data from both *in vitro* and *in vivo* experiments was averaged across all seasons of testing for each month of worm age (Figure 2) and season (Figure 3). *In vitro* viability was higher than *in vivo* for L3 from worms aged 1 month ($p \leq 0.05$) (Figure 2). Across worm age, *in vitro* viability was higher than *in vivo* for the Fall 1 and Winter cycles ($p \leq 0.05$) (Figure 3).

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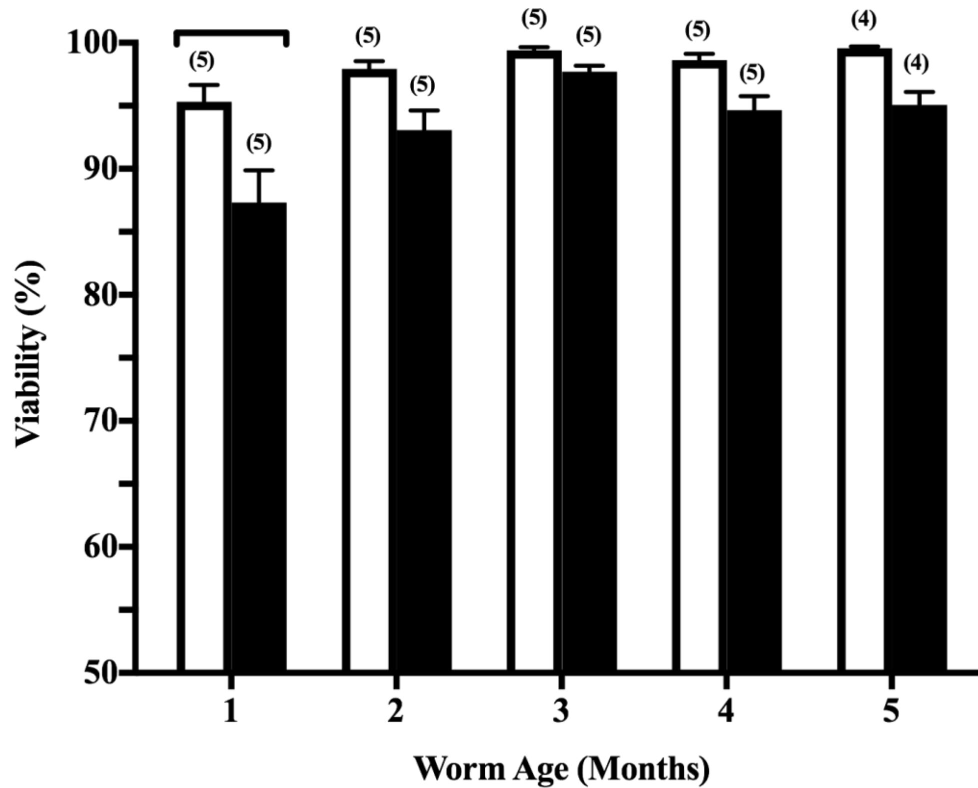

Figure 2. Effect of worm age on percent viability of *Haemonchus contortus* comparing the *in vitro* and *in vivo* methods. Data for the two assays (*in vitro* □ and *in vivo* ■) was averaged across all seasons for each worm age (month). ( ) indicates number of seasonal cycles analyzed for each worm age. Worm age 6 months was excluded from analysis due to insufficient amount of data dictated by infection persistence. All values are Mean ± SEM. Means under bracket differ significantly within the same worm age (months) ($p \leq 0.05$).

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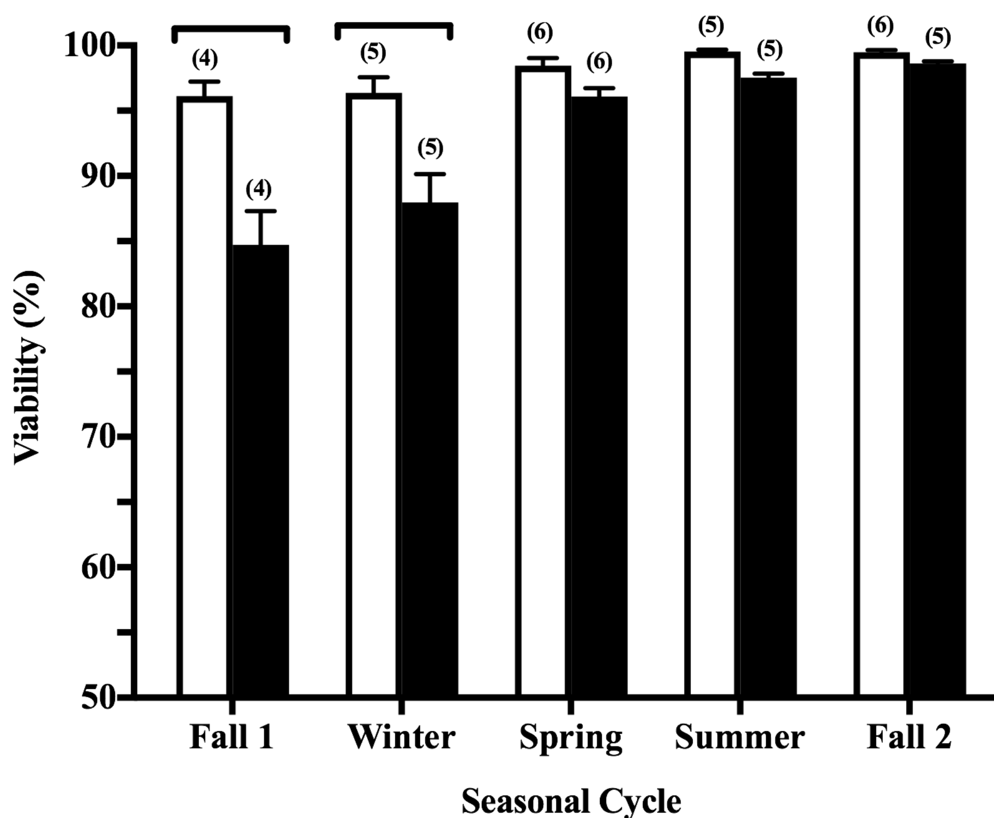

Figure 3. Effect of season on percent viability of *Haemonchus contortus* comparing the *in vitro* and *in vivo.* Data for the two assays (*in vitro* □ and *in vivo* ■) was averaged across all worm ages (months) tested within each season. ( ) indicates number of worm ages (months) analyzed for each seasonal cycle. All values are Mean ± SEM. Means under bracket differ significantly within the same seasonal cycle ($p \leq 0.05$).

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3.6.2 Comparing in vitro vs. in vivo percent live exsheathment

Live exsheathment data from both *in vitro* and *in vivo* experiments was averaged across all seasons of testing for varying worm ages (Figure 4) and season (Figure 5). The mean percent live *in vitro* exsheathment was lower than *in vivo* for worms aged 2 and 4 months ($p \leq 0.05$) (Figure 4). Across season the *in vitro* exsheathment was lower than *in vivo* for seasons Fall 1 and Summer ($p \leq 0.05$) (Figure 5).

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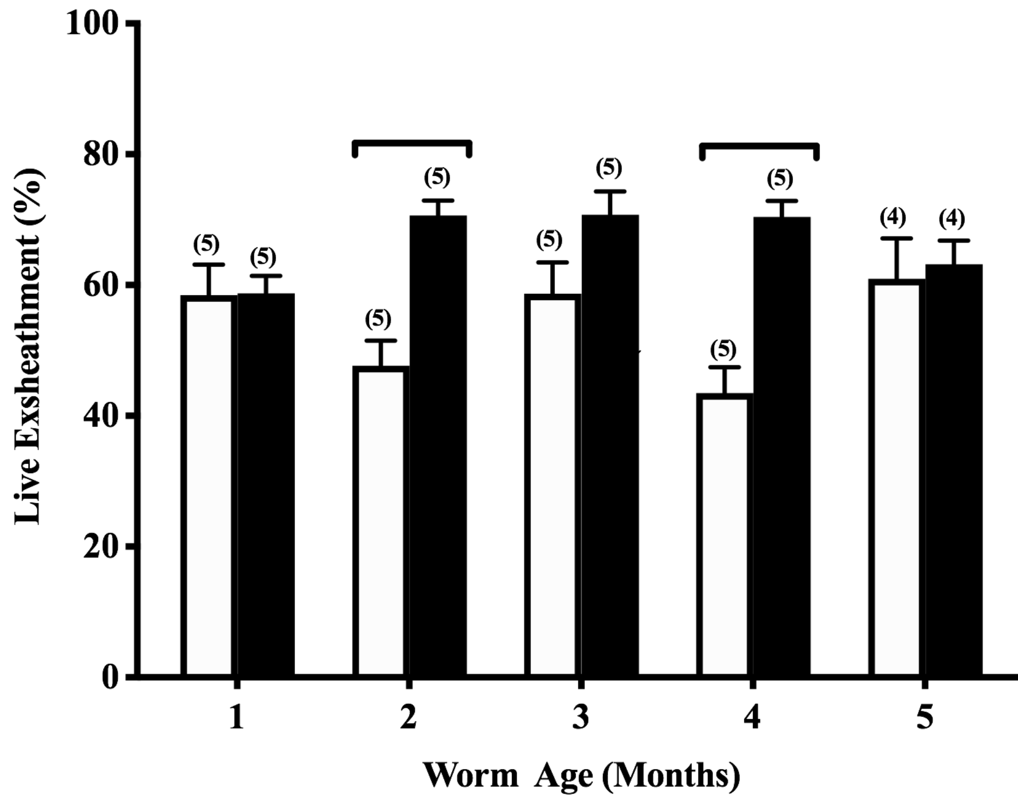

Figure 4. Effect of worm age on percent live exsheathment of *Haemonchus contortus* comparing the *in vitro* and *in vivo* methods. Data for the two assays (*in vitro* □ and *in vivo* ■) was averaged across all seasons for each worm age (month). ( ) indicates number of seasonal cycles analyzed for each worm age. Worm age 6 months was excluded from analysis due to insufficient amount of data dictated by infection persistence. All values are Mean ± SEM. Means under bracket differ significantly within the same worm age (months) ($p \leq 0.05$).

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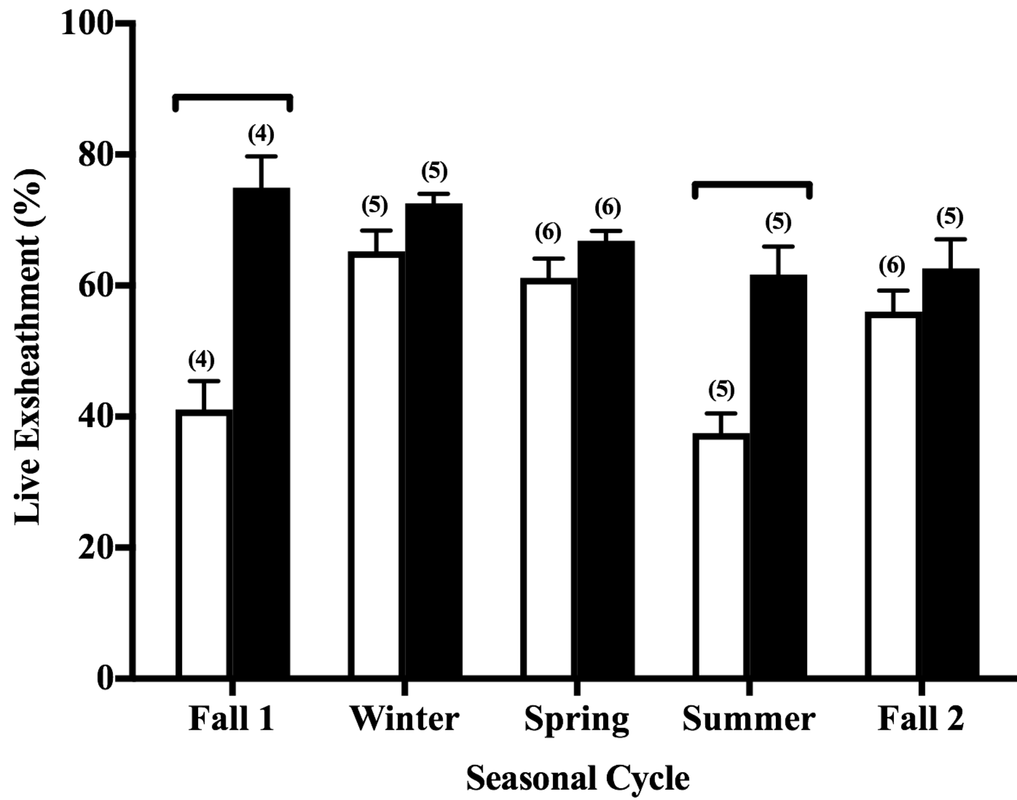

Figure 5. Effect of season on percent live exsheathment of *Haemonchus contortus* comparing the *in vitro* and *in vivo* methods. Data for the two assays (*in vitro* ☐ and *in vivo* ■) was averaged across all worm ages (months) tested within each season. ( ) indicates number of worm ages analyzed for each seasonal cycle. All values are Mean ± SEM. Means under bracket differ significantly within the same seasonal cycle ($p \leq 0.05$).

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4. Discussion:

This study determined that both season and age of the worm producing L3 offspring have an effect on the larvae's ability to exsheath both artificially, *in vitro* as well as within the rumen, *in vivo*. Both season and age of worm also impacted post-assay viability of larvae as well. Although these two factors had an effect on larval exsheathment and viability, there were no apparent effects on egg hatchability. When comparing the two exsheathment assays to one another, it was found that the *in vitro* assay yielded higher viability, but lower exsheathment on average than the *in vivo* assay. Another key difference that was detected between the two assays was the amount of variability for exsheathment results using the *in vitro* assay compared to the *in vivo* assay, especially when looking at the Summer and Fall seasonal cycles.

The method used in the study for assessing exsheathment *in vitro* is a modification of the method proposed by Conder and Johnson (1996) and involves forcing CO_2 gas into tubes containing larvae and 'bubbling' the tubes at 20°C for 15 minutes in order to activate exsheathment and then placing these tubes in an incubator for 18 hours at 37°C (Conder and Johnson, 1996; Barone et al., 2018). Previous work has demonstrated successful exsheathment induction using this method with results indicating >97% larval exsheathment rates (Barone et al., 2018). Other studies using CO_2 as an exsheathment trigger have found that heat shock (rapidly heating larvae to 40 °C) acts in combination with CO_2 to produce high levels of exsheathment (Bekelaar et al., 2018). Bekelaar et al. (2018) found that administering the two triggers simultaneously yielded higher exsheathment rates compared to administering them

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sequentially (simultaneous rates of >75% at 24 hours and 94% exsheathment at 120 hours) (Bekelaar et al., 2018). When the larvae were exposed to CO_2 30 minutes prior to being subjected to heat shock, researchers observed very low levels of exsheathment (<25% at 120 hours) (Bekelaar et al., 2018). These results contradict findings in previous work as well as in the current study where exsheathment rates >90% were observed after just 18 hours for larvae exposed to CO_2 prior to 'heat shock' (incubation at 37°C) (Barone et al., 2018).

Larval exsheathment has been examined *in vivo* in previous studies using a variety of different methods (Sommerville et al., 1957; Hertzberg et al., 2002; Brunet et al., 2007). The containment capsule used in the current study was composed of a piece of tygon tubing capped with two Nunc Cell Culture Inserts (1 at either end of the tube) which is slightly different from those used by Brunet et al. (2007) which were composed of a microtube capped with 1 Nunc Cell Culture Insert. In this previous study (Brunet et al., 2007), researchers were able to achieve exsheathment rates of approximately 80% after 2.7 hours of incubation within the rumen of cannulated sheep, which differs from the results of the current study. Average *in vivo* exsheathment (across all months and seasons of testing) after 8 hours of rumen incubation was ~67% (ranged from 49-90%), which is lower than that which has been previously observed in a shorter amount of time (Brunet et al., 2007). The highest rate for *in vivo* exsheathment (~90%) was observed in larvae from worms aged three months during the Fall 1 cycle. Findings from other *in vivo* studies have been variable in terms of time it takes to achieve high levels of exsheathment, with an older study reporting exsheathment rates of 85% after 5 hours, and a more recent study reporting

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90% exsheathment after just one hour (results that have not been replicated in any *in vivo* work since) (Sommerville et al., 1957; Hertzberg et al., 2002). Previous studies performed within our lab have yielded *in vivo* exsheathment rates >80% after 8 hours of incubation, but exsheathment rate varied greatly between different batches of larvae. In the current study, *in vivo* exsheathment was >75% for multiple timepoints of testing within the different seasons, but this exsheathment rate was not consistent for the duration of the entire trial. Variability in larval exsheathment performance for *H. contortus* has been noted in early *in vivo* studies as well (Sommerville et al., 1957). Factors such as breed/age of fistulated animals, as well as age/source of L3 (donor animal and culture conditions) may contribute to observed differences in *in vivo* exsheathment rates across studies. Hertzberg et al. (2002) used 2-year-old White Alpine Mountain sheep for their study but did not include information on source of L3 (donor animal and culture conditions), but the authors mentioned that larvae used was aged <3 months. Brunet et al. (2007) used Texel sheep (age of sheep not noted), and the authors specified that larvae used in their experiments was obtained from donor goats (no breed is mentioned) and the larvae was aged 2-3 months. Sommerville et al. (1957) does not include information on the age/breed or diet of the fistulated sheep used in their study, nor the age/source of L3 (donor animal and culture conditions) used for experiments. It is important to note that none of these studies included information regarding time of year/season in which the *in vivo* experiments were conducted (Sommerville et al., 1957; Hertzberg et al., 2002; Brunet et al., 2007). The current study used Dorset-cross ewes, 5-6 years old, which are significantly older than the sheep used in the study by Hertzberg et al. (2002). Hertzberg et al. (2002) reported

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that the researchers observed minimal differences in exsheathment rates between different fistulated sheep on trial. Exsheathment rates in the current study did vary between the different ewes (average standard deviation throughout the entire trial was 15 ± 5%), however this would have been a consistent factor throughout the study. Another key difference between this study and previous *in vivo* work is source of L3 used in experiments. Previous studies have reported using larvae 1-3 months old, but the larvae used in our experiments was always freshly harvested L3 from culture (i.e. <2 days old) (Hertzberg et al., 2002; Brunet et al., 2007). Higher exsheathment rates (>80%) have been observed in previous studies, as well as in our own laboratory, all of which used larvae aged 1-3 months (Hertzberg et al., 2002; Brunet et al., 2007). It has been previously suggested that younger L3 may exsheath more readily than older L3 and it has been found that increasing larval age can negatively impact other parameters such as larval migration and motility (Hertzberg et al., 2002; Castaneda-Ramirez et al., 2017). These findings contradict the results of the current study (lower *in vivo* exsheathment rates for younger larvae), but there is a lack of concrete evidence to definitively state that younger larvae will always exsheath more readily than older larvae. More work should be conducted to compare exsheathment rates of fresh collected larvae and aged larvae that has been in storage in order to better understand larval behavior patterns in *in vivo* assays. Due to the lack of consistency and discrepancies in methodology descriptions between previous *in vivo* studies, it is hard to directly compare the results from each study to one another and future work should focus on controlling for the factors outlined above.

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The current study was the first to statistically compare the results using the two specific exsheathment methods (*in vitro* and *in vivo*) and it was found that the *in vitro* and *in vivo* assays yielded different results for both exsheathment rate and post-assay viability rate. The *in vitro* assay had statistically higher post-assay viability results for larvae from worms aged 1 month and for the Fall 1 and Winter cycles when compared to the *in vivo* results, as well as higher viability on average across all seasons and months of testing. These results are due in part to the fact that the *in vitro* assay is tightly controlled and larvae in the tubes are only exposed to water, Earle's Balanced Salt Solution and CO_2, while the larvae in the *in vivo* capsules encounter the rumen environment within a live animal. The opposite trend was observed for exsheathment, with the *in vivo* assay yielding statistically higher exsheathment for larvae from worms aged 2 and 4 months and for the Fall 1 and Summer cycles, as well as higher exsheathment on average across all seasons and months of testing compared to *in vitro*. *In vivo* exsheathment was also more consistent across seasons and worm ages, when compared to *in vitro*, which would indicate that the *in vivo* assay may be more reliable for observing 'normal' exsheathment rates. The greater consistency using the *in vivo* assay may be due to the presence of rumen fluid for the larvae to exsheath in. A previous study focused on testing larval exsheathment responses to temperature change both in CO_2 saturated rumen fluid and CO_2 saturated artificial buffer (Bekelaar et al., 2019). The researchers noted in the results that *H. contortus* larvae showed a significantly lower exsheathment response rate to slow temperature increases in artificial buffer compared to rumen fluid (Bekelaar et al., 2019). The current study compared the *in vitro* and *in vivo* assays to one another strictly in terms of seasonal

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and age of worm effects, but more comparison work can be done to determine how well the assays correlate. It would be interesting for work in the future to compare the assays using different animals (i.e. sheep and goats), different types of infections (experimental vs. natural), different isolated strains of *H. contortus* as well as in different nematode species that exsheath in the rumen such as *O. ostertagi* and *T. circumcincta.*

Variability in *in vitro* exsheathment during the late Summer and early Fall season has been observed in our lab previously (unpublished data). Variation in environmental conditions has been identified as a key factor impacting the life cycle of *H. contortus* (Capitini et al., 1990; Gatongi et al., 1998; Waller et al., 2004; Wang et al., 2014; Wang et al., 2018; Rose et al., 2016). Limited work exists that examines the specific relationship between environment and the exsheathment stage of the *H. contortus* life cycle. It is interesting to note that the season or time of year that studies are performed is typically not reported. In this study, we found there to be a 'seasonal effect' on both *in vitro* and *in vivo* exsheathment and viability, especially for exsheathment during the Fall and Summer cycles. The Winter and Spring cycles yielded more consistent exsheathment rates within and between cycles for both *in vitro* and *in vivo*. The Fall cycles (one and two) both yielded patterns of variability for *in vitro* exsheathment, and even more interestingly, these patterns did not match each other. In both cycles, exsheathment rates showed a pattern of increasing and decreasing between consecutive months of testing, but the exsheathment rates for the individual months did not match between the two cycles. Exsheathment rates for months 1 and 4 differed between the two cycles but were similar to one another in

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months two and three. *In vitro* exsheathment was the lowest (averaged across all months of testing) for the Summer cycle, with exsheathment being <25% for months three, four and five. Donor lambs used for the Summer cycle were infected in late June of 2018 and so *in vitro* and *in vivo* exsheathment assays were performed in late July, August, September, October and November for months one-five of the infection, respectively. Low exsheathment (< 32%) was also observed for the first two months of the Fall 2 cycle, corresponding to assay dates in October and November The patterns of variability observed for the Fall 1, Summer and Fall 1 cycles were not replicated in the *in vivo* assay, which may indicate that the *in vitro* assay itself may be contributing to these observed differences. In order to replicate the observed trends, it would be useful to test different *in vitro* methods (Bahuaud et al., 2006; Bekelaar et al., 2018) during the Fall season using the same batch of larvae, to see if this trend exists using other assays.

Temperature/seasonal factors influencing the host animal's immune response can also have an impact on the infection, and therefore the larval offspring harvested from an infection. Work has been done to examine the relationship between heat stress and susceptibility to *H. contortus* infection, and researchers concluded that susceptible sheep displayed higher stress (high cortisol and FEC) for a longer period of time when compared to sheep bred for resistance (Swarnkar and Singh, 2017). In a follow up study, researchers found there to be a positive relationship between temperature humidity index (THI) and FEC as well as adult worm burden (Swarnkar and Singh, 2018). In the current study, FEC and worm burden were not examined, but the findings in previous studies may indicate that heat stress, as well as THI may influence

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infection development, and therefore the resulting L3 larvae and that this response may be a function of genetic susceptibility to parasitic infection. Future work should focus on testing this hypothesis, as host animal immunity plays a major role in *H. contortus* infection development.

Time in storage (age of larvae) has been identified as a factor influencing *H. contortus* larval exsheathment (Slocombe and Whitlock, 1970; Chyllinksi et al., 2015; Casteñada-Ramírez et al., 2017). The age of the adult worm producing the larvae has yet to be explored as a potential factor influencing exsheathment of the resulting L3 offspring. Research focusing on the relationship between adult worm and larvae offspring is limited for the species *H. contortus* but has been looked at for the commonly studied nematode species, *C. elegans* (Klass et al., 1977). There is an overwhelming amount of work that has been done to examine *C. elegans* as a model for parasitic nematodes, which has been reviewed by different authors several times within the last 20 years (Hashmi et al., 2001; Geary and Thompson et al., 2001; Gilleard, 2004). For the species *C. elegans*, it has been found that parental (adult worm) age influences progeny lifespan, with progeny from younger worms showing greater mean lifespans (days) over time, compared to those from older worms (Klass et al., 1977). Similarly, it was found that parental (adult worm) lifespan had a direct impact on progeny lifespan, again with progeny from worms with longer lifespans producing offspring with greater lifespans compared to those with parental worms with shorter lifespans (Klass et al., 1977). The relationship between maternal adult *C. elegans* and resulting offspring has been furthered studied and it has been determined that maternal age influences the phenotype of the progeny, with young maternal adults

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(1 day old) producing progeny with a lower 'fitness' (Perez et al., 2017). It is hypothesized that the differences observed between progeny from young and old maternal adult worms may be caused by age-dependent alterations in the formation of the lipoprotein complex vitellogenin within the embryo (Perez et al., 2017). Similar work has been done with other invertebrate species including *B. manjavacas* and *D. melanogaster,* supporting the hypothesis that maternal age can have a negative impact on offspring fitness and viability (Bock et al., 2019; Bloch et al., 2017). Although these patterns have not been explored in the species *H. contortus* specifically, there is an abundance of evidence to support the notion that parental age can influence traits in resulting offspring. The results of this study both support and contradict findings from previous studies in other species. The current study found there to be various differences in *in vitro* exsheathment between larvae from adult worms of different ages, with cycles Spring and Summer showing a negative correlation with adult worm age. The opposite was seen for *in vitro* and *in vivo* viability, with cycles Fall 1, Winter and Spring all yielding lower viability for larvae from worms aged 1 month compared to larvae from older worms. These findings indicate that age of an infection in a donor animal used for research can influence larvae performance in different exsheathment assays and should be considered when crafting the study design of the study.

This study was designed to examine the effects of season and age of the adult worm on larval exsheathment. Other factors, however, that could potentially be contributing to the observed differences, could not be controlled for in the statistical model. Animal resources were not available that would have enabled us to control for genetic relatedness, gender, as well as age within each of the different seasons

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therefore a decision was made to maximize, to the extent possible, the genetic relatedness between the donor animals used. It has been previously determined that age of host animal can influence larval development in trichostrongyle infection, with mature ewes demonstrating a lower development of larvae to L3 stage when compared to development patterns in 3-month-old lambs (Jorgensen et al., 1998). Due to higher susceptibility to *H. contortus* infection in young animals, lambs < 9 months old are typically used in research involving *H. contortus* experimental infection (Gonzalez et al., 2008; Schichowski et al., 2010; Katiki et al., 2012; Gressler et al., 2014; Tonin et al., 2014). The current study used lambs aged 6 months for the Fall 1, Spring and Fall 2 cycles, and lambs aged 9 months for the Winter and Summer cycles. Research has been conducted to compare immune responses of lambs aged 6 and 9 months (Hohenhaus et al., 1995; Kooyman et al., 2000). One particular study found that antibody titers against *H. contortus* increased significantly between 6 and 9 months of age (Hohenhaus et al., 1995). Similarly, another study determined that lambs aged 6 months were 6% less protected against *H. contortus* infection post-vaccination when compared to 9-month-old lambs (Kooyman et al., 2000). In the current study, fecal egg counts were not statistically analyzed, but it is important to mention that on average, 6-month-old lambs yielded higher fecal egg counts across seasonal cycles compared to 9-month-old lambs. This finding is consistent with that of previous studies that 6-month-old lambs display a lower immune response to *H. contortus* compared to 9-month-old lambs. It is clear that immune responses of lambs aged 6 and 9 months do differ, therefore the age of the donor animal should be controlled for in future studies as a potential factor influencing larval behavior in experimental assays.

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It has also been determined that host immunity can have an impact on adult female nematode fecundity in GIN species (Strain et al. 2002; Sargison et al., 2011). In the current study, there were differences in infection persistence (a measure of fecundity) between the different seasonal cycles, with the Spring and Fall 2 cycles persisting to 6 months (6-month-old donor lambs) and the Winter and Summer cycles persisting to 5 months (9-month-old donor lambs). Interestingly, the Fall 1 cycle also used 6-month-old donor lambs and did not persist past 4 months. To date, there are no studies that explicitly examine how donor lamb age could influence larval performance in exsheathment assays, and so future exsheathment studies (*in vitro* and *in vivo*) should examine lamb age in more depth.

As previously mentioned, donor lamb sex could not be controlled for in the statistical model. Genetic relatedness was a key priority in selecting donor lambs for infection and so gender differed across seasons with the Fall 1, Winter and Fall 2 cycles using male donor lambs and the Spring and Summer cycles using female donor lambs. Previous work has been conducted to compare male and female lambs in terms of *H. contortus* infection development patterns (Barger, 1993; Luffau et al., 1981; Adams, 1989; Albers et al., 1989; Woollaston et al., 1990; Shaw et al., 1995), but findings from these studies contradict one another. While some studies report male lambs as generally more susceptible to nematode infection when compared to females (Barger, 1993; Luffau et al., 1981; Adams, 1989), other studies report no consistent differences between the two genders (Albers et al., 1989; Woollaston et al., 1990). One particular study even reported that males on trial had significantly lower fecal egg counts than females on trial, and the researchers noted that this finding contradicts

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research within the same field (Shaw et al., 1995). More recent work focusing on *Teladorsagia circumcincta,* found that male lambs on trial had higher fecal egg counts and higher adult worm burden at necropsy when compared to female lambs, but the authors mentioned that this difference may be due to male lambs ingesting more grass (i.e. more larvae) (Abuargob and Stear, 2014). Future studies are needed to determine the effect of donor lamb gender on exsheathment of resulting L3.

Although it was not outlined as a main objective, this study uncovered variability in L3 development patterns. Culture periods varied between 7-14 days across different seasons. In our laboratory, the standard protocol for culturing larvae involves a 14-day incubation of fecal matter at room temperature (~23°C). Prior to the start of the trial, this protocol was modified to a 7-day incubation period at room temperature due to observations of low viability (<90%) upon harvest of L3 after 14 days. There is a considerable amount of variability in *H. contortus* culture protocols across previously conducted studies, with some reporting a culture period of 5 days at 28°C and others, 14 days at 23°C (Casteñada-Ramírez et al., 2017; Bekelaar et al., 2018). There are multiple studies that do not describe/specify culturing procedures and so the exact time period for L3 development is unknown for these studies (Alonso-Diaz et al., 2008; von Son-de Fernex et al., 2012; Klongsiriwet et al., 2015; Barone et al., 2018). It is known that temperature and humidity have a direct impact on the development of trichostrongylid nematodes (Pandey et al., 1989; Rossanigo and Gruner, 1995). Cultures in this study were incubated at room temperature, but were not kept in temperature controlled incubators and so temperatures conditions did fluctuate with a range of 18-26°C. It is possible that small fluctuations in temperature

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could have lead to changes in development patterns of larvae, but other factors, particularly genetic factors, may be responsible for the observed differences. This study observed differences in development time period for larvae from same sex twin donor lambs, as well as the previously mentioned differences in average fecal egg counts and patterns of infection persistence. Although there is a large body of work that has been done to study genetic factors influencing *H. contortus* infection particularly in terms of susceptibility and/or resistance to GIN infection in host animals (Nieuwoudt et al., 2002; Marshall et al., 2009; Alba-Hurtado et al., 2010; Estrada et al., 2016), future studies are needed to determine the impact of host animal genetics on larval development under laboratory culture conditions. Further research is needed to standardize laboratory culture protocols, including control for donor genetics, gender and immune status as well as laboratory environmental conditions.

5. Conclusion

This study was the first to monitor and compare exsheathment patterns of *Haemonchus contortus* both *in vitro* and *in vivo* according to all four seasons in order to uncover patterns associated with seasonality. This study also examined the effect of age of the adult worm producing larvae offspring that are used in exsheathment assays and how this may play a role in larval performance. Evidence for an effect of worm age*season was apparent for larval exsheathment as well as viability both *in vitro* and *in vivo* but was not observed for egg hatchability. Upon comparing the *in vitro* and *in vivo* assays to one another, a pattern for higher *in vitro* viability, but lower exsheathment was uncovered. Another key finding was the evident variability in the

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exsheathment results for the *in vitro* assay, particularly during the Fall and Summer cycles. The results of the current study should be explored further in order to confirm the specific roles that seasonality and age of adult worm have on *H. contortus* viability and exsheathment. Ultimately, this study was able to uncover new information that can inform future research efforts to combat GIN infection in small ruminants.

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CHAPTER III

FUTURE RESEARCH DIRECTIONS

Introduction:

There were three main objectives outlined for this research. The first objective was to test the effects of both season as well as *H. contortus* worm age on hatchability of resulting eggs *in vitro.* The second objective was to test the effects of the same two factors on viability of resulting larvae (L3) and their ability to undergo exsheathment both *in vitro* using CO_2 and *in vivo* in rumen fistulated ewes. The final objective of the study was to statistically compare results from the *in vitro* and *in vivo* exsheathment assays to determine how well they correlate to one another. For the first objective of the research, it was determined that neither season nor age of worm had any impact on hatchability of *H. contortus in vitro.* For the second objective, it was found that both season and worm age had an impact on viability and exsheathment of *H. contortus* L3 *in vitro* and *in vivo*. For the final objective, differences between the *in vitro* and *in vivo* assay were detected according to both effect or season and worm age. In addition to the primary findings of the study, this work uncovered information about larval development patterns in culture, with some larvae developing to L3 stage in 7 days and other samples requiring a 14-day period to reach L3 stage. More research is needed to explore the effects of seasonal changes and worm age on larval performance in different experimental assays. Additionally, exsheathment studies in the future should focus on comparing different exsheathment assays (*in vitro* and *in vivo*) to one another in order to uncover trends for how well assays correlate. Lastly, it is important to fully understand larval development patterns in culture and why certain batches of

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larvae develop quicker than others, so more work is needed to confirm the findings in the current study. These areas for future research are described in more detail below.

Variability of larval exsheathment according to seasonal changes and worm age:

A considerable amount of variability in exsheathment rates was observed for the Fall 1, Summer and Fall 2 cycles. The observed variability in the Fall 1 cycle called for the addition of the Fall 2 cycle to the trial, in order to see if the pattern of variability repeated itself. Interestingly, the Fall 2 cycle yielded variable exsheathment, but the exact pattern observed in the first Fall cycle was not replicated. Both cycles were characterized by alternating rates of exsheathment (high and low) in consecutive months, but the cycles differed from one another in terms of the rates observed for each individual month of testing. In the Summer cycle, exsheathment started out high (~81%) and steadily decreased over the five months of testing (~17% in month five). These patterns of variability were not seen in the *in vivo* assay, which would indicate that perhaps the *in vitro* assay itself may be contributing to these observed differences, in addition to seasonal factors. This hypothesis should be tested in future research involving the comparison of multiple *in vitro* assays during different seasons using larvae from worms of different ages to see if any variability patterns can be detected using other assays. It would be ideal for future studies to control for donor lamb age and sex to eliminate the potential influence these factors may have on *in vitro* exsheathment.

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Comparing *in vitro* and *in vivo* exsheathment assays:

In comparing the two exsheathment assays (*in vitro* and *in vivo*) to one another, it was found that the *in vitro* assay tended to yield higher post-assay viability, but lower exsheathment, on average, compared to that of the *in vivo* assay. Statistically significant differences in viability between the two assays were observed for larvae from worms aged one month (across all seasons) and for Fall 1 and Winter (across all worm ages). For exsheathment, significant differences between the two assays were observed for larvae from worms aged two and four months (across all seasons) and for the Fall 1 and Summer cycles (across all worm ages). Future exsheathment studies should focus on comparing *in vitro* and *in vivo* exsheathment assays to one another in order to determine if the assays are accurately assessing larval exsheathment. In addition to comparing the assays to one another in general, more work is needed to explore the influence of season and worm age and how these factors may be affecting larval exsheathment differently in *in vitro* and *in vivo* assays. The two specific exsheathment assays used in this study are not widely used in this field, and so more work using these exact methods is required to test their reliability.

Variability in larval development to L3 under different culture conditions:

Development patterns of *H. contortus* eggs to L3 stage differed according to seasonal cycle. While larvae developed to L3 stage at room temperature in just 7 days for the Fall 1 cycle and the majority of the Winter cycle, larvae failed to develop at this rate for the Spring, Summer and Fall 2 cycles and required more time (9-14 days) to do so. What is even more intriguing, is that the development patterns of larvae from related donor lambs used within the same cycle differed from one another. For

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example, in the Spring cycle, larvae from one twin donor lamb took longer to develop (14 days) than larvae from the other twin donor lamb (9 days). This is also true for the Fall 2 cycle, with one donor lamb producing larvae that developed in culture to L3 stage as early as 7 days, while larvae from the other twin required a 14-day culture incubation. Individual host infection factors, such as genetic susceptibility to GIN, differences in egg laying and/or development or differences within the abomasal environment between donor animals should researched in more depth in future work. In addition to exploring individual host factors, more studies should examine season and how it may play a role in the speed with which larvae reach L3 stage in culture.

Conclusion:

More work is needed to test seasonality and age of the adult worm as factors influencing larval exsheathment *in vitro* and *in vivo*. This study has shown that these parameters have a negative impact on larval exsheathment during certain seasons and according to different worm ages *in vitro* and *in vivo*, and so it is important for future work to continue this line of research. The exact mechanism for how these factors act together in order to influence larval exsheathment should become a priority in future *H. contortus* research. Additionally, in order to effectively study the exsheathment stage of the *H. contortus* life cycle, *in vitro* and *in vivo* assays should yield results comparable to one another. Tailoring future work to involve direct comparison of different assays can further refine methodology for how the *H. contortus* life cycle is studied. Lastly, an improved understanding of how *H. contortus* larvae develops in culture can enhance future gastrointestinal nematode (GIN) control research efforts.

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APPENDIX 1

Standard Operating Procedure for Blood Collection

Supplies:

- $[K^2]$EDTA (lavender top) blood collection tubes
- 21g, 1.5" Vacutainer needles
- Vacutainer holder
- Cooler and tube rack

Procedure:

1. Properly restrain animal holding the head parallel to the ground and at a 30° angle to the side.
2. Electric clippers may be used to prepare the neck by shaving off a patch of wool approximately 4 inches wide and 8 inches long.
3. To hold off the vein, apply pressure with the thumb approximately half way down the side of the neck (on either side). The vein can be visualized and palpated once filled with blood.
4. Secure a Vacutainer needle to the Vacutainer holder by twisting in.
5. While holding off on the vein with one hand, insert the needle into the vein with the bevel facing upward.
6. Once the needle is placed, push the EDTA tube into the holder and onto the needle.

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7. Gently adjust the needle until blood begins to flow into the tube. Blood flow will cease once the tube is full.
8. Gently remove the tube and slowly invert eight times.
9. Remove the needle and your hand from the neck and allow vein to relax.
10. EDTA tubes should be immediately placed in ice.

(Adapted protocol from the thesis of Caitlyn MacGlaflin; modified from Purdue University Blood Sampling in Sheep by Mitchell et al. (Retrieved on September 23, 2008 from http://www.ces.purdue.edu/extmedia/AS/AS-557-W.pdf)

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APPENDIX 2

Standard Operating Procedure for Determining Packed Cell Volume

Supplies:

- Hematocrit capillary tubes
- Capillary tube sealing wax
- Kim wipes
- PCV reader

Procedure:

1. Collect blood by jugular venipuncture into Vacutainer EDTA tubes, invert tube eight times, and place on ice.
2. Invert tube to mix before use.
3. Without spilling, tip tube so that blood moves toward the opening. Place one end of the micro hematocrit capillary tube into the blood and allow tube to fill by capillary action. Only fill tube up to ¾ of the way.
4. Place fingertip at one end of the tube to prevent leaking. Insert the other end of the tube into the sealing wax. Wipe off the tube with a kim wipe if there is any blood on the outside.
5. Place capillary tubes into centrifuge rotor, making sure that the wax in the end of the tube faces outward (not towards the center of the centrifuge). All samples should be run in duplicate and placed across from each other, keeping the centrifuge balanced.
6. To prevent tubes from cracking, take a pin and gently push tubes against the outer edge of the rotor.

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7. Centrifuge for 3 minutes at 15,000 RPM at room temperature.

Sample Reading:

1. Spin the PCV Reader until it reaches the 100 mark.
2. Place the capillary tube into the sample groove.
3. Align the wax/blood interface with the line at the bottom of the sample groove.
4. Spin the top plate of the PCV reader until the “swirl” aligns with the plasma/air interface.
5. Spin both plates until the “swirl” aligns with the white blood cell/red blood cell interface. The value under the red line is the PCV to be read and reported as a percentage.

(Adapted protocol from the thesis of Caitlyn MacGlaflin)

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APPENDIX 3

Packed Cell Volume Data for All Trial Cycles

	Fall 1 Donors			Winter Donors			Spring Donors			Summer Donors			Fall 2 Donors	
	1704	1705		1706	1707		H1820	H1821		H1824	H1829		1829	1833
Date			Date			Date			Date			Date		
9/18/2017	34	33	12/18/2017	37	36	3/26/2018	33	30	6/25/2018	32	33	9/24/2018	32	28
9/25/2017	27	30	12/25/2017	38	34	4/2/2018	33	30	7/2/2018	30	30	10/1/2018	32	31
10/2/2017	31	32	1/1/2018	37	35	4/9/2018	30	26	7/9/2018	30	29	10/8/2018	30	26
10/9/2017	29	29	1/8/2018	30	32	4/16/2018	31	24	7/16/2018	29	29	10/15/2018	27	24
10/16/2017	23	27	1/15/2018	30	27	4/23/2018	30	23	7/23/2018	25	25	10/22/2018	25	23
10/23/2017	26	28	1/22/2018	27	27	4/30/2018	32	25	7/30/2018	29	29	10/29/2018	25	27
10/30/2017	27	27	1/29/2018	28	26	5/7/2018	31	25	8/6/2018	30	27	11/5/2018	25	25
11/6/2017	28	27	2/5/2018	29	26	5/14/2018	29	24	8/13/2018	28	27	11/12/2018	25	24
11/13/2017	26	24	2/12/2018	30	26	5/21/2018	26	23	8/20/2018	28	27	11/19/2018	26	25
11/20/2017	29	26	2/19/2018	28	26	5/28/2018	28	25	8/27/2018	34	29	11/26/2018	25	26
11/27/2017	25	26	2/26/2018	29	28	6/4/2018	27	23	9/3/2018	32	33	12/3/2018	25	25
12/4/2017	29	28	3/5/2018	30	26	6/11/2018	30	26	9/10/2018	31	27	12/10/2018	29	25
12/11/2017	27	26	3/12/2018	30	27	6/18/2018	29	24	9/17/2018	31	-	12/17/2018	27	22
12/18/2017	30	30	3/19/2018	33	29	6/25/2018	26	21	9/24/2018	30	28	12/24/2018	-	-
12/25/2017	32	29	3/26/2018	30	39	7/2/2018	28	23	10/1/2018	29	31	12/31/2018	30	26
1/1/2018	28	29	4/2/2018	32	29	7/9/2018	29	28	10/8/2018	33	33	1/7/2019	24	27
1/8/2018	29	28	4/9/2018	32	27	7/16/2018	27	21	10/15/2018	32	33	1/14/2019	27	29
1/15/2018	27	28	4/16/2018	31	30	7/23/2018	31	21	10/22/2018	35	-	1/21/2019	25	27
1/22/2018	30	28	4/23/2018	30	30	7/30/2018	32	25	10/29/2018	32	34	1/28/2019	27	29
1/29/2018	30	29	4/30/2018	33	29	8/6/2018	29	30	11/5/2018	32	25	2/4/2019	25	27
2/5/2018	26	28	5/7/2018	36	31	8/13/2018	32	22	11/13/2018	30	30	2/11/2019	-	-
			5/14/2018	34	31	8/20/2018	31	24	11/19/2018	30	34	2/18/2019	26	25
			5/21/2018	24	32	8/27/2018	31	25	11/26/2018	36	34	2/25/2019	28	28
			5/28/2018	30	35	9/3/2018	28	26	12/3/2018	32	34	3/4/2019	28	29
			6/4/2018	-	30	9/10/2018	-	-	12/10/2018	31	37	3/11/2019	29	30

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APPENDIX 4

Standard Operating Procedure for Fecal Egg Counts

Supplies:

- Small cups (2 for each sample)
- Cheese cloth (double-ply, cut into 3”x3” squares)
- Tongue depressors
- Transfer pipets
- McMaster slide(s)

Procedure:

1. Fecal samples are collected directly from the rectum and kept refrigerated until analysis. Samples should be run as promptly as possible, but within seven days from the time of collection.
2. Two grams of feces are measured on a calibrated scale and placed into a l-2 ounce cup.
3. Add 28 ml Fecasol® to feces and soak for approximately 5 minutes.
4. Gently break up fecal pellet with a tongue depressor. Set cup aside to sit for approximately 5 minutes.
5. Pour fecal solution through a square of 2-ply cheesecloth into a new cup. Use tongue depressor to gently press fecal solution through gauze.
6. Wet the McMaster slide with distilled water and gently pat dry top and bottom with paper towels.

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7. Immediately pipet solution into both sides of the McMaster slide, using a 1 ml syringe, a sample of the suspension and fill one side of the chamber.
8. Place slide on microscope platform and let sit, without disturbance, for 5 minutes.
9. Focus on the top layer using the low power (10x) objective. Count all eggs inside of the grid areas (greater than ½ of egg inside grid).
10. Count only trichostrongylid eggs (oval shaped, ~80-90 microns long)
11. Total egg count:

$$(\text{chamber } 1 + \text{chamber } 2) * 50 = \text{eggs per gram (epg)}$$

(Whitlock (1948), Modified McMaster Technique)

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APPENDIX 5

Fecal Egg Count Data for All Trial Cycles

	Fall 1 Donors			Winter Donors			Spring Donors			Summer Donors			Fall 2 Donors	
	1704	1705		1706	1707		H1820	H1821		H1824	H1829		1829	1833
Date			Date			Date			Date			Date		
9/18/2017	0	50	12/18/2017	0	100	3/19/2018	0	0	6/25/2018	0	0	9/17/2018	0	0
9/25/2017	0	0	12/25/2017	0	50	3/26/2018	0	0	7/2/2018	0	50	9/24/2018	0	0
10/2/2017	0	0	1/1/2018	0	0	4/2/2018	0	0	7/9/2018	1450	50	10/1/2018	50	0
10/9/2017	0	0	1/8/2018	200	1000	4/9/2018	0	50	7/16/2018	4750	900	10/8/2018	50	0
10/16/2017	1800	2550	1/15/2018	5850	4750	4/16/2018	1250	1400	7/23/2018	7650	3550	10/15/2018	3000	950
10/23/2017	4950	6200	1/22/2018	4050	3100	4/23/2018	2800	4200	7/30/2018	5650	3000	10/22/2018	3750	2700
10/30/2017	5400	7900	1/29/2018	6000	4300	4/30/2018	3750	4550	8/6/2018	6100	3050	10/29/2018	6350	3050
11/6/2017	3800	4150	2/5/2018	5250	5050	5/7/2018	4000	4150	8/13/2018	4350	5000	11/5/2018	5200	3200
11/13/2017	4200	4500	2/12/2018	5350	3750	5/14/2018	3800	4700	8/20/2018	6350	5800	11/12/2018	2950	2200
11/20/2017	3650	3800	2/19/2018	2950	3000	5/21/2018	4800	4800	8/27/2018	7700	6050	11/19/2018	3750	2750
11/27/2017	2900	7650	2/26/2018	7550	4050	5/28/2018	3950	3800	9/3/2018	6100	7100	11/26/2018	6100	5200
12/4/2017	1550	2500	3/5/2018	4100	2700	6/4/2018	4200	4900	9/10/2018	2100	2200	12/3/2018	3150	2600
12/11/2017	700	2750	3/12/2018	2900	2800	6/11/2018	4600	5600	9/17/2018	2650	850	12/10/2018	4800	3250
12/18/2017	350	1950	3/19/2018	4100	2350	6/18/2018	4300	7650	9/24/2018	950	2200	12/17/2018	5050	2600
12/25/2017	200	1500	3/26/2018	2850	1400	6/25/2018	3100	7450	10/1/2018	1150	1250	12/24/2018	-	-
1/1/2018	200	650	4/2/2018	900	1200	7/2/2018	3650	4600	10/8/2018	850	150	12/31/2018	5600	1450
1/8/2018	100	1000	4/9/2018	1750	1200	7/9/2018	2750	3950	10/15/2018	800	150	1/7/2019	4200	350
1/15/2018	100	850	4/16/2018	1100	1300	7/16/2018	1850	3350	10/22/2018	300	250	1/14/2019	4850	850
1/22/2018	0	100	4/23/2018	700	850	7/23/2018	1550	4000	10/29/2018	350	300	1/21/2019	4650	900
1/29/2018	50	50	4/30/2018	600	650	7/30/2018	1900	2200	11/5/2018	200	200	1/28/2019	5150	900
2/5/2018	0	0	5/7/2018	150	550	8/6/2018	1600	1850	11/13/2018	250	300	2/4/2019	6250	1100
			5/14/2018	100	650	8/13/2018	950	3050	11/19/2018	350	300	2/11/2019	5050	900
			5/21/2018	0	400	8/20/2018	600	2000	11/26/2018	200	250	2/18/2019	5250	950
			5/28/2018	0	300	8/27/2018	1350	2800	12/3/2018	100	50	2/25/2019	6750	750
			6/4/2018	0	350	9/3/2018	800	3550	12/10/2018	100	100	3/4/2019	6850	650
						9/10/2018	350	2950				3/11/2019	7050	550

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APPENDIX 6

Cleaning cannulated ewes and removing cannulas

Supplies:

- Stand
- Halter
- Water source (buckets of warm water during winter, hose during summer)
- Blow drier or towels (cold weather only)
- Gloves
- Dawn soap
- Electric clippers (when needed)
- Bug repellent during fly season such as CLAC (Deo Lotion)

Procedure:

1. Halter ewe and put her on the stand.
2. Put on gloves.
3. Thoroughly soak the dirty area around the cannula (weather permitting).
4. Lift the flap of the cannula and remove the caked-on rumen debris.
5. Place soap on your hand and rub it into the wool to further loosen debris.
6. When all of the rumen debris is loose, rinse off the soap.
7. Wipe excess water off the ewe with gloved hand.
8. During cold weather, dry with blow drier or towel.

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9. If needed, clip the wool around and under the cannula flap.

10. During fly season, spread repellent on the wool around and on the cannula

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APPENDIX 7

Standard Operating Procedure for Egg Recovery

Supplies:

- Small cup (4oz/120cc)
- Nitrile gloves
- 1mm, 355µm, 150µm, 38µm, and 25µm sieves
- 15ml and 50ml Falcon™ tubes
- Fecasol®
- Glass cover slips
- Transfer pipets
- 20µL Micropipette with tips

Procedure:

1. Obtain 8-10 grams of feces from animal with more than 2,000 epg.
2. Place feces in small cup (4oz/120cc).
3. Activate hatchability.
    a. Add enough water to break up the feces.
    b. Mush with hands. End with slurry.
    c. Rinse hand.
4. Rinse through 1mm sieve

. Place sieve over empty bucket (11 liter bucket).

a. Pour mixture through sieve, rubbing with hand to increase speed.

b. Rub and rinse through with tap water until clear.

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c. Rinse sieve in sink.

d. Run mixture through sieve again without rinsing, discard remaining debris.

5. Repeat step 4 through the 355µm and150µm sieve.

6. Rinse through the 38µm sieve but do not discard solids collected in sieve. This now contains eggs. Use water to rinse the solids off the sieve into an empty bucket.

    . Collect the water that was rinsed through the 38µm sieve into four or more 50ml Falcon™ tubes to be used during the egg hatch assay.

7. Repeat step 6 through the 25µm sieve. Use 50ml Fecasol® to rinse solids into a large glass beaker.

8. Use transfer pipets to evenly distribute egg mixture into four 15ml Falcon™ tubes. Using Fecasol®, bring each volume to the top of the tube to form a positive meniscus. Carefully place a cover slip on the top of the tube and place in the centrifuge.

9. Using the centrifuge, spin tubes at 200 x g for 2 minutes.

10. Slowly remove cover slips by lifting straight up off the top of the tubes and rinse with water over the 25µm sieve.

11. Wash off the 25µm sieve with 14mL of tap water into beaker. Pipette into 15ml Falcon™ tube.

12. Using the micropipette, measure out 10µL and put on a slide. Determine the concentration of eggs in the 14mL of water and calculate a total number of eggs.

    1. Obtain 5 or more egg counts of 10µL pulls and calculate an average. The Falcon™ tube should be inverted 5-8 times prior to obtaining each pull.

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Ex: if there are an average of 69 eggs in 10μL

69 eggs10μL=x14,000μL→10x=966,000→x=96,600 eggs [in 14mL]

(Adapted protocol from Miller parasitology laboratory, unpublished methods)

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APPENDIX 8

Standard Operating Procedure for Egg Hatch and L1 Mortality Assay

(Adapted protocol from Assis et al. (2003) and Marie-Magdeleine et al. (2009); *in vitro* methods)

<u>Procedure:</u>

1. Weigh out desired amount of either PAC or plant material. Create stock solution using tap water, determining the stock solution concentration to be double of the highest concentration in the wells. Prepare serial dilutions of extracts using tap water.
2. Make control solution using Thiabendazole (TBZ, Thermo Fisher Scientific Inc., Waltham, MA, USA) in dimethyl sulfoxide (DMSO, Fisher BioReagents™, Thermo Fisher Scientific Inc., Waltham, MA, USA; REF #BP231-1) at a concentration of 1 mg TBZ/mL DMSO (1.4 mg TBZ dissolved in 1400 µL DMSO).
3. Obtain a concentration of eggs from recovery. Correct concentration of egg solution to be approximately 100 eggs in 100 µl.
4. Add 100 eggs (in 100 µl) per well into a 24-well plate (Corning™, Falcon™, Polystyrene Microplates, Corning Life Sciences, Tewksbury, MA, USA; REF #353226).
5. Designate negative controls by only adding tap water to wells. Add 10µl of the TBZ stock solution to each positive control well (in a total volume of 2 mL this will give a final concentration of 0.5 µg/mL of TBZ).
6. For wells that did not get 10 µl of TBZ solution, add 10 µl of DMSO.

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7. All wells receive 890 µl tap water increasing the volume to 1 mL.
8. Add either prepared PAC extract solutions (1 ml) or add 1 ml water to negative or positive control wells, increasing to total volume of 2 mL.
9. Incubate for 24 hours at 26ºC.
10. Count number of eggs and live larvae in each well (based on 5 second observation of motility) at 24 hours.

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APPENDIX 9

Egg Hatch Data for All Trial Cycles

Assay Date	Seasonal Cycle	Age of Infection (Months)	Donor (Replicate)	Egg Hatchability (%)	Notes
10/24/2017	1	1	1704	96	
	1	1	1705	97.78	
11/20/2017	1	2	1704	98.2	
	1	2	1705	97.5	
12/11/2017	1	3	1704	99.01	
	1	3	1705	98.49	
1/15/2018	1	4	1704	N/A	Note 1
	1	4	1705	95.82	
1/23/2018	2	1	1706	97	
	2	1	1707	97.5	
2/20/2018	2	2	1706	98	
	2	2	1707	95.53	
3/20/2018	2	3	1706	98.07	
	2	3	1707	97.5	
4/17/2018	2	4	1706	98.43	
	2	4	1707	98.63	
4/24/2018	3	1	H1820	96.57	
	3	1	H1821	95.88	
5/21/2018	3	2	H1820	92.93	
	3	2	H1821	94.82	
6/19/2018	3	3	H1820	96.6	
	3	3	H1821	96.06	
7/19/2018	3	4	H1820	96.9	
	3	4	H1821	96.46	
8/14/2018	3	5	H1820	98.31	
	3	5	H1821	96.96	
9/10/2018	3	6	H1820	96.82	
	3	6	H1821	96.56	
7/24/2018	4	1	H1824	96.11	
	4	1	H1829	95.89	
8/20/2018	4	2	H1824	97.03	
	4	2	H1829	97.66	
9/18/2018	4	3	H1824	86.18	
	4	3	H1829	95.3	
10/16/2018	4	4	H1824	90.95	
	4	4	H1829	96.14	
11/14/2018	4	5	H1824	94.78	
	4	5	H1829	98.01	
10/23/2018	5	1	1829	96.27	
	5	1	1833	92.23	
11/19/2018	5	2	1829	96.19	
	5	2	1833	95.14	
12/10/2018	5	3	1829	97.16	
	5	3	1833	98.17	
1/14/2018	5	4	1829	97.96	
	5	4	1833	97.91	
2/12/2019	5	5	1829	97.72	
	5	5	1833	98.04	
3/12/2019	5	6	1829	N/A	Note 1
	5	6	1833	96.05	

Notes	
Note 1	FEC for one donor was too low to perform egg recovery

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APPENDIX 10

Standard Operating Procedure for L3 Mortality and Exsheathment Inhibition Assay

(Adapted protocol from Conder and Johnson (1996), *in vitro* methods)

Procedure:

1. Obtain L_3 infective larvae, less than 3 months old
2. Sheathed L3 larvae (2,000) are added to Earle's Balanced Salt Solution (EBSS, Sigma-Aldrich®, Inc., Natick, MA, USA; REF #E3024) up to a volume of 1 mL in a 15 mL Falcon™ tube.
3. Tap water (1 mL) will be added to water control larvae, for a total volume of 2 mL in the Falcon™ tube. Larvae will be checked for viability
4. Parafilm M® (Bemis Company, Inc., Neenah, WI; REF #HS234526A-1) is used to stretch over the top of the tubes as a cover. A glass pipet tip, connected to a CO_2 tank, is carefully placed down into the larval solution by puncturing through the Parafilm M®. Making sure the Parafilm M® is still stretched.
5. CO_2 is then bubbled into tubes for 15 minutes immediately prior to the incubation period.
6. Caps are screwed on each tube for incubation after removing the CO_2 feeder tube.
7. Tubes are then placed back into the incubator at 38°C for 18-24 hours.

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8. Following the 18-hour incubation, percent exsheathment and percent viability are determined by averaging counts of exsheathed larvae from the first 100 motile worms observed.

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APPENDIX 11

In vitro Exsheathment and Viability Data for All Trial Cycles

Assay Date	Seasonal Cycle	Age of Infection (months)	Donor	Replicate	Viability	Adjusted Viability	Exsheathment	Adjusted Exsheathment	Notes
10/31/2017	1	1	1704	No CO_2	96		7.5		Note 1
	1	1	1704	CO_2 A	82	86.3	88.0	87.0	
	1	1	1704	CO_2 B	87	91.2	47.1	42.8	
11/27/2017	1	2	1704	No CO_2	96		19.1		
	1	2	1704	CO_2 A	93	96.8	40.9	27.0	
	1	2	1704	CO_2 B	95	98.5	36.0	21.0	
	1	2	1705	No CO_2	95		3.3		
	1	2	1705	CO_2 A	86	91.3	22.4	19.8	
	1	2	1705	CO_2 B	94	99.3	31.1	28.7	
12/18/2017	1	3	1704	No CO_2	98		4.8		
	1	3	1704	CO_2 A	98	99.9	72.5	71.1	
	1	3	1704	CO_2 B	97	98.9	89.0	88.5	
	1	3	1705	No CO_2	100		0.9		
	1	3	1705	CO_2 A	100	100.0	52.6	52.2	
	1	3	1705	CO_2 B	95	94.7	63.5	63.2	
	1	3	1705	CO_2 C	100	100.0	64.2	63.9	
1/22/2018	1	4	1704	No CO_2	66		0.0		
	1	4	1704	CO_2 A	61	93.1	28.7	28.7	
	1	4	1704	CO_2 B	57	87.4	17.1	17.1	
	1	4	1704	CO_2 C	63	96.4	21.5	21.5	
	1	4	1705	No CO_2	31		0.0		
	1	4	1705	CO_2 A	55	100.0	20.2	20.2	
	1	4	1705	CO_2 B	64	100.0	26.1	26.1	
	1	4	1705	CO_2 C	57	100.0	19.8	19.8	

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Assay Date	Seasonal Cycle	Age of Infection (months)	Donor	Replicate	Viability	Adjusted Viability	Exsheathment	Adjusted Exsheathment	Notes
1/29/2018	2	1	1707	No CO_2	90		3.7		
	2	1	1707	CO_2 A	88	97.3	55.5	53.7	
	2	1	1707	CO_2 B	83	91.7	57.7	56.0	
	2	1	1707	CO_2 C	86	94.8	55.8	54.0	
	2	1	1706	No CO_2	87		6.3		
	2	1	1706	CO_2 A	62	71.8	74.0	72.3	
	2	1	1706	CO_2 B	76	88.1	74.8	73.1	
	2	1	1706	CO_2 C	78	89.8	68.6	66.5	
2/26/2018	2	2	1707	No CO_2	90		22.1		
	2	2	1707	CO_2 A	98	100.0	73.8	66.4	
	2	2	1707	CO_2 B	98	100.0	77.6	71.2	
	2	2	1707	CO_2 C	98	100.0	79.4	73.5	
	2	2	1706	No CO_2	98		16.4		
	2	2	1706	CO_2 A	94	95.2	75.0	70.1	
	2	2	1706	CO_2 B	95	96.3	66.4	59.8	
	2	2	1706	CO_2 C	86	87.7	64.2	57.1	
3/27/2018	2	3	1707	No CO_2	97		2.9		Note 2
	2	3	1707	CO_2 A	100	100.0	73.3	72.5	
	2	3	1707	CO_2 B	95	97.5	74.3	73.5	
	2	3	1707	CO_2 C	97	99.5	62.2	61.1	
4/24/2018	2	4	1707	No CO_2	95		0.8		
	2	4	1707	CO_2 A	97	100.0	65.3	65.0	
	2	4	1707	CO_2 B	98	100.0	56.9	56.5	
	2	4	1707	CO_2 C	94	98.4	75.7	75.5	
	2	4	1706	No CO_2	98		0.9		
	2	4	1706	CO_2 A	97	98.3	62.2	61.9	
	2	4	1706	CO_2 B	98	100.0	59.5	59.1	
	2	4	1706	CO_2 C	96	97.8	57.1	56.7	
5/21/2018	2	5	1707	No CO_2	90		5.9		
	2	5	1707	CO_2 A	97	100.0	73.9	72.2	
	2	5	1707	CO_2 B	93	100.0	61.0	58.6	
	2	5	1707	CO_2 C	91	100.0	75.8	74.3	
	2	5	1706	No CO_2	95		0.9		
	2	5	1706	CO_2 A	93	97.7	85.6	85.5	
	2	5	1706	CO_2 B	98	100.0	52.2	51.8	
	2	5	1706	CO_2 C	95	100.0	64.0	63.7	

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Assay Date	Seasonal Cycle	Age of Infection (months)	Donor	Replicate	Viability	Adjusted Viability	Exsheathment	Adjusted Exsheathment	Notes
5/9/2018	3	1	H1820	No CO_2	92		0.9		
	3	1	H1820	CO_2 A	86	94.1	67.7	67.4	
	3	1	H1820	CO_2 B	95	100.0	65.4	65.1	
	3	1	H1820	CO_2 C	89	97.4	78.3	78.1	
	3	1	H1821	No CO_2	86		0.0		
	3	1	H1821	CO_2 A	92	100.0	60.3	60.3	
	3	1	H1821	CO_2 B	80	93.0	68.0	68.0	
	3	1	H1821	CO_2 C	73	84.2	66.9	66.9	
5/29/2018	3	2	H1820	No CO_2	89		7.4		Note 2
	3	2	H1820	CO_2 A	94	100.0	81.6	80.1	
	3	2	H1820	CO_2 B	96	100.0	66.0	63.3	
	3	2	H1820	CO_2 C	92	100.0	61.9	58.9	
7/3/2018	3	3	H1820	No CO_2	98		0.9		Note 2
	3	3	H1820	CO_2 A	97	98.9	68.9	68.6	
	3	3	H1820	CO_2 B	99	100.0	61.5	61.1	
	3	3	H1820	CO_2 C	97	99.0	69.9	69.6	
7/31/2018	3	4	H1820	No CO_2	96		2.5		
	3	4	H1820	CO_2 A	98	100.0	61.8	60.8	
	3	4	H1820	CO_2 B	97	100.0	66.7	65.8	
	3	4	H1820	CO_2 C	97	100.0	58.1	57.0	
	3	4	H1821	No CO_2	99		4.6		
	3	4	H1821	CO_2 A	97	97.4	54.7	52.5	
	3	4	H1821	CO_2 B	98	98.7	62.4	60.6	
	3	4	H1821	CO_2 C	97	97.9	52.9	50.7	
8/29/2018	3	5	H1820	No CO_2	99		0.0		
	3	5	H1820	CO_2 A	98	98.9	53.3	53.3	
	3	5	H1820	CO_2 B	98	99.3	55.4	55.4	
	3	5	H1820	CO_2 C	98	99.0	53.6	53.6	
	3	5	H1821	No CO_2	96		0.0		
	3	5	H1821	CO_2 A	99	100.0	79.4	79.4	
	3	5	H1821	CO_2 B	99	100.0	74.3	74.3	
	3	5	H1821	CO_2 C	97	100.0	59.1	59.1	
9/25/2018	3	6	H1820	No CO_2	100		0.0		
	3	6	H1820	CO_2 A	99	99.0	55.3	55.3	
	3	6	H1820	CO_2 B	100	100.0	52.0	52.0	
	3	6	H1820	CO_2 C	98	97.8	40.7	40.7	
	3	6	H1821	No CO_2	99		0.0		
	3	6	H1821	CO_2 A	98	99.1	55.1	55.1	
	3	6	H1821	CO_2 B	100	100.0	46.1	46.1	
	3	6	H1821	CO_2 C	99	100.0	55.9	55.9	

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Assay Date	Seasonal Cycle	Age of Infection (months)	Donor	Replicate	Viability	Adjusted Viability	Exsheathment	Adjusted Exsheathment	Notes
8/8/2018	4	1	H1824	No CO_2	74		0.0		
	4	1	H1824	CO_2 A	80	100.0	73.5	73.5	
	4	1	H1824	CO_2 B	86	100.0	80.5	80.5	
	4	1	H1824	CO_2 C	89	100.0	68.8	68.8	
	4	1	H1829	No CO_2	92		1.9		
	4	1	H1829	CO_2 A	94	100.0	86.8	86.5	
	4	1	H1829	CO_2 B	98	100.0	84.3	84.0	
	4	1	H1829	CO_2 C	93	100.0	91.4	91.2	
9/5/2018	4	2	H1824	No CO_2	99		0.9		
	4	2	H1824	CO_2 A	97	98.0	76.0	75.8	
	4	2	H1824	CO_2 B	100	100.0	52.9	52.5	
	4	2	H1824	CO_2 C	99	100.0	45.1	44.6	
	4	2	H1829	No CO_2	100		0.0		
	4	2	H1829	CO_2 A	96	96.4	50.0	50.0	
	4	2	H1829	CO_2 B	99	99.2	46.0	46.0	
	4	2	H1829	CO_2 C	98	97.6	38.7	38.7	
10/3/2018	4	3	H1824	No CO_2	95		0.0		
	4	3	H1824	CO_2 A	95	100.0	21.7	21.7	
	4	3	H1824	CO_2 B	96	100.0	18.1	18.1	
	4	3	H1824	CO_2 C	93	98.6	32.9	32.9	
	4	3	H1829	No CO_2	97		0.0		
	4	3	H1829	CO_2 A	99	100.0	20.3	20.3	
	4	3	H1829	CO_2 B	96	99.3	23.1	23.1	
	4	3	H1829	CO_2 C	97	100.0	26.3	26.3	
10/30/2018	4	4	H1824	No CO_2	100		0.0		
	4	4	H1824	CO_2 A	100	100.0	19.4	19.4	
	4	4	H1824	CO_2 B	99	99.2	9.8	9.8	
	4	4	H1824	CO_2 C	100	100.0	15.8	15.8	
	4	4	H1829	No CO_2	99		0.0		
	4	4	H1829	CO_2 A	100	100.0	15.5	15.5	
	4	4	H1829	CO_2 B	100	100.0	10.8	10.8	
	4	4	H1829	CO_2 C	100	100.0	16.9	16.9	
11/27/2018	4	5	H1824	No CO_2	98		0.0		
	4	5	H1824	CO_2 A	97	98.8	20.6	20.6	
	4	5	H1824	CO_2 B	100	100.0	10.9	10.9	
	4	5	H1824	CO_2 C	98	99.7	16.7	16.7	
	4	5	H1829	No CO_2	97		0.0		
	4	5	H1829	CO_2 A	98	100.0	15.9	15.9	
	4	5	H1829	CO_2 B	99	100.0	17.1	17.1	
	4	5	H1829	CO_2 C	96	99.0	21.1	21.1	

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Assay Date	Seasonal Cycle	Age of Infection (months)	Donor	Replicate	Viability	Adjusted Viability	Exsheathment	Adjusted Exsheathment	Notes
11/7/2018	5	1	1833	No CO_2	97		0.0		
	5	1	1833	CO_2 A	99	100.0	17.9	17.9	
	5	1	1833	CO_2 B	99	100.0	17.2	17.2	
	5	1	1833	CO_2 C	98	100.0	15.1	15.1	
	5	1	1829	No CO_2	97		0.9		
	5	1	1829	CO_2 A	96	98.7	29.3	28.6	
	5	1	1829	CO_2 B	99	100.0	23.9	23.2	
	5	1	1829	CO_2 C	98	100.0	22.4	21.7	
12/5/2018	5	2	1833	No CO_2	93		0.0		
	5	2	1833	CO_2 A	89	95.8	29.4	29.4	
	5	2	1833	CO_2 B	91	97.7	22.1	22.1	
	5	2	1833	CO_2 C	92	98.8	22.0	22.0	
	5	2	1829	No CO_2	77		0.0		
	5	2	1829	CO_2 A	84	100.0	42.1	42.1	
	5	2	1829	CO_2 B	94	100.0	33.6	33.6	
	5	2	1829	CO_2 C	93	100.0	38.0	38.0	
1/1/2019	5	3	1833	No CO_2	100		0.0		
	5	3	1833	CO_2 A	100	100.0	88.2	88.2	
	5	3	1833	CO_2 B	100	100.0	74.8	74.8	
	5	3	1833	CO_2 C	100	100.0	69.0	69.0	
	5	3	1829	No CO_2	99		0.0		
	5	3	1829	CO_2 A	99	100.0	73.1	73.1	
	5	3	1829	CO_2 B	100	100.0	79.7	79.7	
	5	3	1829	CO_2 C	100	100.0	77.3	77.3	
1/30/2019	5	4	1833	No CO_2	96		0.0		
	5	4	1833	CO_2 A	97	100.0	45.8	45.8	
	5	4	1833	CO_2 B	98	100.0	66.1	66.1	
	5	4	1833	CO_2 C	100	100.0	59.1	59.1	
	5	4	1829	No CO_2	100		0.0		
	5	4	1829	CO_2 A	99	99.1	67.9	67.9	
	5	4	1829	CO_2 B	97	97.3	63.9	63.9	
	5	4	1829	CO_2 C	98	98.3	58.4	58.4	
2/27/2019	5	5	1833	No CO_2	100		1.0		
	5	5	1833	CO_2 A	100	100.0	97.1	97.1	
	5	5	1833	CO_2 B	100	100.0	98.1	98.1	
	5	5	1833	CO_2 C	100	100.0	96.2	96.1	
	5	5	1829	No CO_2	100		0.0		
	5	5	1829	CO_2 A	98	98.2	95.4	95.4	
	5	5	1829	CO_2 B	99	99.1	95.3	95.3	
	5	5	1829	CO_2 C	100	100.0	98.3	98.3	
3/27/2019	5	6	1833	No CO_2	100		0.0		Note 3
	5	6	1833	CO_2 A	100	100.0	57.0	57.0	
	5	6	1833	CO_2 B	100	100.0	39.6	39.6	
	5	6	1833	CO_2 C	100	100.0	37.4	37.4	

Notes	
Note 1	Larvae recovery for one donor failed (due to low viability coming out of culture)
Note 2	Larvae recovery for one donor failed (<95% L3 coming out of culture)
Note 3	Larvae recovery for one donor failed (due to low FEC)

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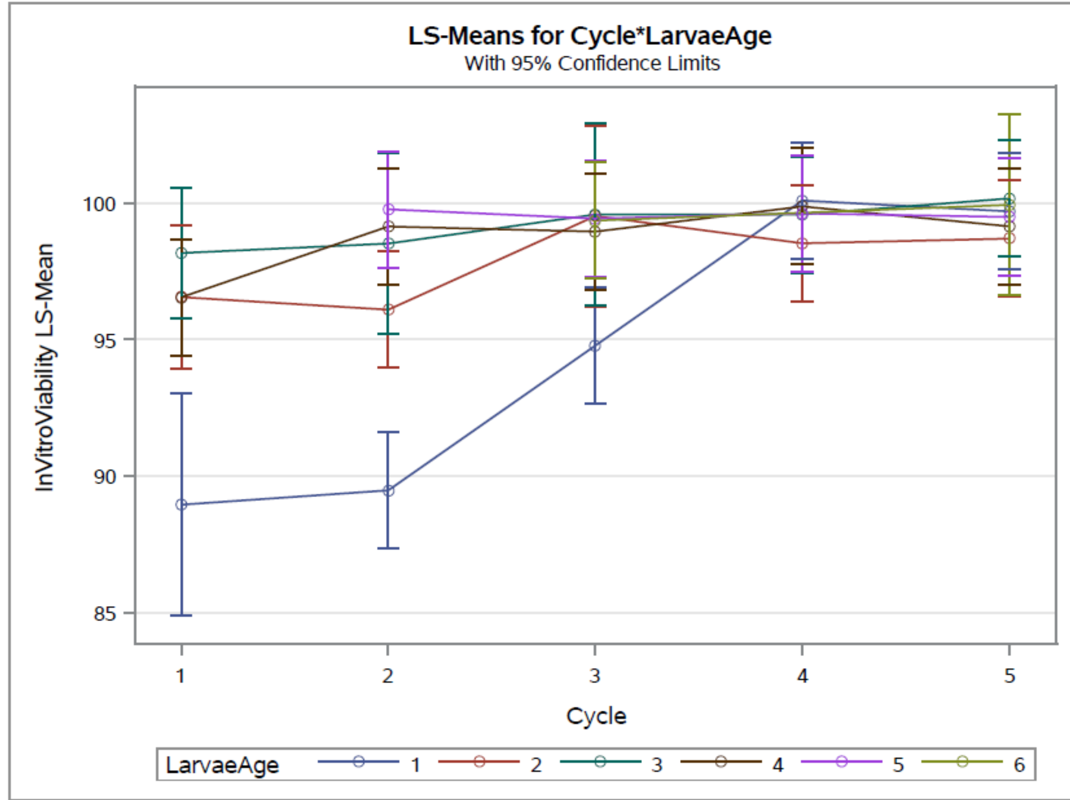
APPENDIX 12

Graph of *In vitro* Exsheathment Assay Viability Results



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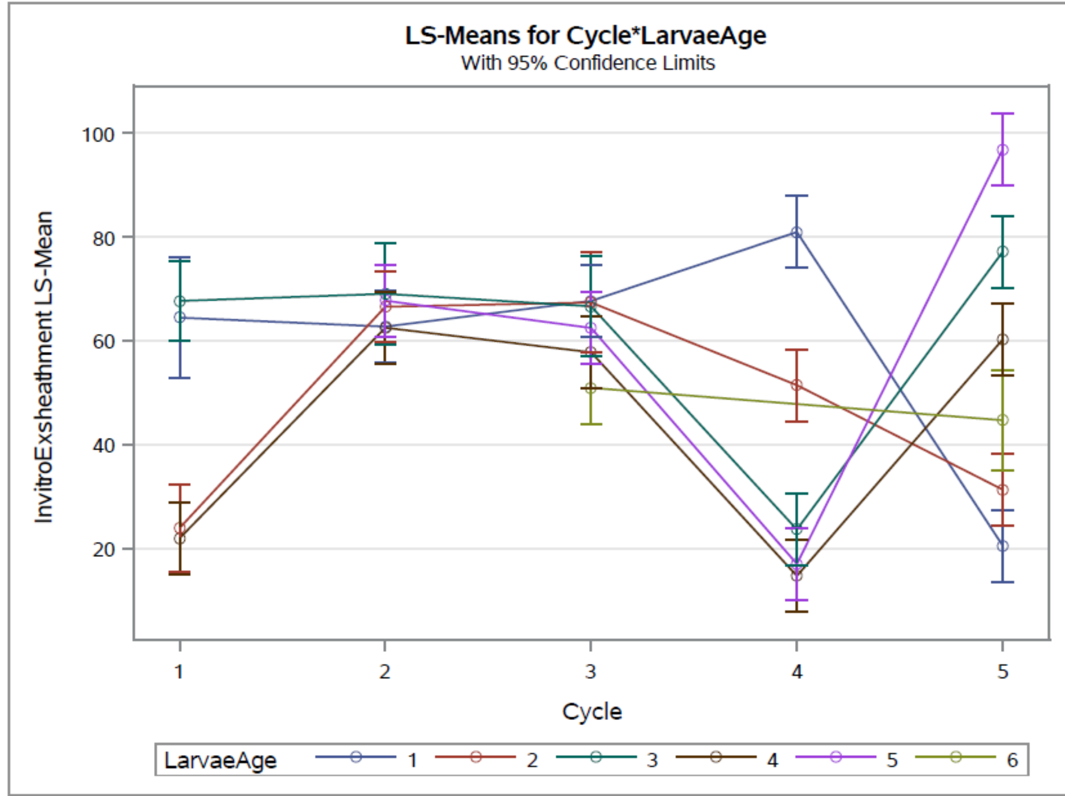
APPENDIX 13

Graph of *In vitro* Exsheathment Assay Exsheathment Results



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APPENDIX 14

In vivo Exsheathment Inhibition and L3 Mortality Assay

Supplies:

- 20,000 *H. contortus* larvae
- Microscope
- Slides
- 20 µL pipette and tips
- 1000 µL pipette and tips
- Eight 3.8 cm pieces of Tygon® tubing; ID: 9.5 mm OD: 14.3 mm (Fisher Scientific, Hampton, NH).
- 16 Nunc™ Cell Culture Inserts (i.e. Nunc™ top)
- 4 Cannula plugs with two 20 cm cords attached to the inner side of each
- 16 small zip ties
- 3 mL syringe and 25 G needle
- Thermometer
- 3 large buckets
- 8 labeled 2mL capsules
- Paper towels
- 8 5x10 cm heat sealed concentrate bags (R510, ANKOM Technology, Macedon, NY)
- Impulse heat sealer
- Shoulder length gloves

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- Gloves
- 4 rumen fistulated ewes
- 4 halters
- Movable panel
- Thermo water heater
- Small scissors
- Small labeled cups (labeled with ear tag numbers)
- Tube rocker

<u>Procedure:</u>

Day before Experiment:

1. Set larvae out at room temperature 20-24 hours before start of experiment.
2. Read approximate concentration of motile ensheathed larvae (MEnL).
    - Determine # MEnL per 1µL (divide average MEnL by 10).
    - Determine # µL necessary to = 2000 larvae (divide 2000 by #MEnL per 1 µL).

Day of Experiment:

3. Fill one bucket with very warm tap water.

4. Insert one Nunc™ top into one end of each Tygon® tube. Top should be at least 3/4ths covered by tubing.

5. Place all tubes into the bucket filled with very warm tap water to soften tubes.

6. Fill other another bucket with 37ºC tap water (use thermometer).

7. Remove first tube from very warm tap water.

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8. Using 1000 μL pipette to pipette the number of μLs necessary to equal 2000 larvae (determined previous day) into softened tube.

9. Pipette 37ºC water into softened tube until approximately 2/3rds full.

10. Carefully insert second Nunc™ top into open end of tube.

11. Place in 37ºC water after seal is made and push Nunc™ top into tube so that 3/4ths is covered by the tubing.

12. Using the syringe and needle, insert the needle into the capsule near the middle of the capsule, but at a nearly parallel angle so that the needle enters the inner part of the tube near one of the Nunc™ tops.

13. Submerge at least one end of the capsule underwater in the 37ºC water and draw back on the syringe to remove the air pocket. The goal is to make the air pocket as small as possible without removing any liquid containing the larvae.

14. Leave the completed capsule in the water and repeat steps 7-13 until all eight capsules are completed.

15. Fill another large bucket half way with 37ºC water.

16. Remove a capsule from the water and dry the outsides using paper towels.

17. Place in one heat seal-able bag and seal end using impulse sealer.

18. Using two zip ties, attach the capsule to one end of a cannula plug string.

- Wrap one zip tie around the tube and bag and through the loop on the cannula plug string; tighten the zip tie to a snug position.
- Repeat with the second zip tie.

19. Repeat steps 16-18 until all capsules are attached. When a cannula plug has both capsules attached, submerge in the fresh bucket of 37ºC water.

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20. Bring down to fistulated ewes: bucket with cannulas and capsules, shoulder length gloves, and regular gloves.

21. Use movable panel and halters to catch and secure the four fistulated sheep.

22. Remove cannula plug from sheep with lowest ear-tag number and insert capsules.

- Using arm with shoulder length glove, cup capsules in hand and insert as deep as possible into rumen.
- Orient strings to be at the bottom of the U-bolt they are tied to.
- Insert cannula plug and orient so that the outer U- bolt is parallel to the ground (this makes the inner U-bolt perpendicular to the ground).

23. Repeat for the rest of the fistulated sheep in the order of increasing ear-tag numbers (ex: 1206, 1301, 1308, 1314).

24. Note time of first inserted capsule (Generally 7-8am).

25. Release the ewes and give them their morning feeding.

26. Rinse regular cannula plugs and place somewhere where their smell won't bother others.

27. Read remaining larvae used for set-up to determine pre-experiment motility and exsheathment percentages.

- Look at a minimum of 150 motile larvae.
- Be sure to record the age of the larvae and other flask information.

28. Clean-up from set-up.

29. Get together afternoon supplies

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- Fill empty bucket with: more shoulder length gloves, extra regular gloves, 4 halters, small scissors, and labeled cups.
- label the eight 2mL capsules with ear tag numbers (two for each sheep).

30. Wait determined amount of time (8 hours) and remove capsules (3-4pm depending on start time).
31. Approximately 30 minutes before removing capsules:
    - Turn on the Thermo water heater to 37ºC (confirm temp with thermometer).
    - Fill bucket with 37ºC tap water.
32. Bring supplies in bucket from step 29 and the bucket with water to sheep.
33. Dump half the 37ºC water in with the cannula plugs that were removed that morning to soften the plugs.
34. Dump half the remaining water into the labeled cups.
35. Catch the sheep with the movable panel and tie them using the halters.
36. Remove capsules starting with sheep with lowest ear-tag number.
    - When removing capsules avoid pulling out by the strings. Instead reach into rumen with gloved hand, cup capsules, and remove gently.
37. Replace cannula plug with plain plugs from the morning.
38. Cut and discard both zip ties and cut heat sealed bag off of larvae capsule.
39. Rinse capsule in remaining water and place in appropriately labeled cup.
40. Repeat steps 36-40 for all four sheep.
41. Release sheep and clean the dirty cannula plugs and attached strings.

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42. Discard rumen-fluid-covered gloves/heatsealed bags/etc in dumpster to avoid attracting flies.

43. Using small scissors, cut several indents in one end of the capsule's tube.

44. Remove Nunc^{TM} top and pour larvae containing fluid into appropriately labeled 2mL capsule.

45. Place capsule into 37ºC water in water heater.

46. Repeat steps 43-46 for all capsules.

47. Place each 2mL capsule on tube rocker when ready to read

48. Read larvae:

- Using 20uL pipette read 10uL drops of larvae at a time
- Keep track of exsheathed motile/non-motile and ensheathed motile/non-motile
- Read until 150 motile larvae or 200 total larvae (whichever comes first).
- Calculate % Motility (viability) and % Exsheathment
- % Motile (viable) = Total motile/Total
- For exsheathment calculations only motile larvae are included.

% Exsheathment = (# Motile Exsheathed/#Total Motile) x 100%

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APPENDIX 15

In vivo Exsheathment and Viability Data for All Trial Cycles

Assay Date	Season/Cycle	Age of Infection (months)	Donor	Replicate (Fistulated Ewe)	% Viability	% Live Exsheathment	Notes
10/31/2017	1	1	1704	1206	54.1	85.53	Note 1
	1	1	1704	1301	65	71.43	
	1	1	1704	1308	82	52.41	
	1	1	1704	1314	73.6	40.9	
11/28/2017	1	2	1704	1206	67	74.56	Note 3
	1	2	1704	1308	63.05	56.69	
	1	2	1704	1314	69.1	60.24	
	1	2	1705	1206	92.96	67.57	
	1	2	1705	1301	95	54.39	
	1	2	1705	1308	92	85.63	
	1	2	1705	1314	84.9	81.5	
12/19/2017	1	3	1704	1206	85.1	85	
	1	3	1704	1301	98	86.1	
	1	3	1704	1308	94.5	84.97	
	1	3	1704	1314	100	100	
	1	3	1705	1206	98.86	93.1	
	1	3	1705	1301	98.9	96.69	
	1	3	1705	1308	97.4	93.5	
	1	3	1705	1314	95.5	81.9	
1/23/2018	1	4	1704	1206	74.88	59.21	
	1	4	1704	1301	78.35	27.63	
	1	4	1704	1308	72	80.1	
	1	4	1704	1314	76.3	87.4	
	1	4	1705	1206	96.8	95	
	1	4	1705	1301	93.8	80.3	
	1	4	1705	1308	91.62	63.41	
	1	4	1705	1314	96.99	78.26	

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Assay Date	Season/Cycle	Age of Infection (months)	Donor	Replicate (Fistulated Ewe)	% Viability	% Live Exsheathment	Notes
1/30/2018	2	1	1706	1206	78.2	76.14	
	2	1	1706	1301	81.57	72.3	
	2	1	1706	1308	63.7	79.8	
	2	1	1706	1314	80.53	83.01	
	2	1	1707	1206	84.6	56.6	
	2	1	1707	1301	56.27	54.78	
	2	1	1707	1308	42	64.8	
	2	1	1707	1314	68.55	72.35	
2/27/2018	2	2	1706	1206	91.14	84.72	
	2	2	1706	1301	93.9	64.5	
	2	2	1706	1308	96.2	86.8	
	2	2	1706	1314	98.4	44.9	
	2	2	1707	1206	98.15	82.39	
	2	2	1707	1301	98.7	82.1	
	2	2	1707	1308	99.4	71.8	
	2	2	1707	1314	99.6	74.5	
3/27/2018	2	3	1707	1206	96.47	73.17	Note 2
	2	3	1707	1301	95.7	73.1	
	2	3	1707	1308	97.6	85.29	
	2	3	1707	1314	97.5	21	
4/24/2018	2	4	1706	1206	94.38	70.24	
	2	4	1706	1301	92.26	67.74	
	2	4	1706	1308	94.86	75.9	
	2	4	1706	1314	95.43	87.43	
	2	4	1707	1206	94.76	87.09	
	2	4	1707	1301	92	62.73	
	2	4	1707	1308	90.86	96.86	
	2	4	1707	1314	94.34	43.3	
5/22/2018	2	5	1706	1206	90.6	85.2	
	2	5	1706	1301	84.9	94.9	
	2	5	1706	1308	94.74	94.44	
	2	5	1706	1314	95.52	56.25	
	2	5	1707	1206	90.39	58.13	
	2	5	1707	1301	80.77	55.95	
	2	5	1707	1308	87.08	91.21	
	2	5	1707	1314	76.3	81.3	

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Assay Date	Season/Cycle	Age of Infection (months)	Donor	Replicate (Fistulated Ewe)	% Viability	% Live Exsheathment	Notes
5/1/2018	3	1	H1820	1206	95.6	23.6	
	3	1	H1820	1301	93.6	64.8	
	3	1	H1820	1308	94	59.4	
	3	1	H1820	1314	92.97	69.19	
	3	1	H1821	1206	91.58	64.34	
	3	1	H1821	1301	82.16	68.42	
	3	1	H1821	1308	91.54	68.48	
	3	1	H1821	1314	95.83	89.44	
5/29/2018	3	2	H1820	1206	94.61	65.19	Note 2
	3	2	H1820	1301	84.6	77.5	
	3	2	H1820	1308	94.44	83.01	
	3	2	H1820	1314	88.2	84.5	
7/4/2018	3	3	H1820	1206	99.49	63.59	Note 2
	3	3	H1820	1301	98.91	68.86	
	3	3	H1820	1308	100	73.1	
	3	3	H1820	1314	98.77	93.13	
7/31/2018	3	4	H1820	1206	96.67	46.55	
	3	4	H1820	1301	97.62	54.88	
	3	4	H1820	1308	98.7	64.47	
	3	4	H1820	1314	96.39	58.29	
	3	4	H1821	1206	97.95	58.64	
	3	4	H1821	1301	99.53	57.55	
	3	4	H1821	1308	98.7	46.71	
	3	4	H1821	1314	95.86	62.35	
8/29/2018	3	5	H1820	1206	97.3	32.78	
	3	5	H1820	1301	95.75	71.11	
	3	5	H1820	1308	98.71	80.39	
	3	5	H1820	1314	99.01	68.5	
	3	5	H1821	1206	96.17	36.36	
	3	5	H1821	1301	96.97	76.88	
	3	5	H1821	1308	97.44	67.76	
	3	5	H1821	1314	99.47	79.03	
9/26/2018	3	6	H1820	1206	98.81	42.17	
	3	6	H1820	1301	100	67.55	
	3	6	H1820	1308	97.63	88.48	
	3	6	H1820	1314	97.31	82.32	
	3	6	H1821	1206	98.25	58.04	
	3	6	H1821	1301	96.32	72.13	
	3	6	H1821	1308	97.81	93.85	
	3	6	H1821	1314	99.4	90.42	

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Assay Date	Season/Cycle	Age of Infection (months)	Donor	Replicate (Fistulated Ewe)	% Viability	% Live Exsheathment	Notes
8/7/2018	4	1	H1824	1206	95.29	42.31	
	4	1	H1824	1301	97.11	61.31	
	4	1	H1824	1308	96.95	32.7	
	4	1	H1824	1314	100	61.82	
	4	1	H1829	1206	97.57	44.55	
	4	1	H1829	1301	100	74.17	
	4	1	H1829	1308	98.77	37.27	
	4	1	H1829	1314	97.7	54.12	
9/5/2018	4	2	H1824	1206	95.7	68.54	
	4	2	H1824	1301	95.91	70.12	
	4	2	H1824	1308	90.17	85.89	
	4	2	H1824	1314	97.17	53.91	
	4	2	H1829	1206	98.15	57.86	
	4	2	H1829	1301	99.39	74.85	
	4	2	H1829	1308	98.03	83.92	
	4	2	H1829	1314	98.76	83.02	
10/3/2018	4	3	H1824	1206	98.7	64.47	
	4	3	H1824	1301	96.04	41.24	
	4	3	H1824	1308	96.32	59.56	
	4	3	H1824	1314	98.09	59.51	
	4	3	H1829	1206	98.11	66.83	
	4	3	H1829	1301	99.41	98.22	
	4	3	H1829	1308	99.35	59.74	
	4	3	H1829	1314	98.47	44.04	
10/30/2018	4	4	H1824	1206	98.24	65.87	
	4	4	H1824	1301	96.2	85.53	
	4	4	H1824	1308	99.11	81.61	
	4	4	H1824	1314	98.94	83.42	
	4	4	H1829	1206	98.79	66.87	
	4	4	H1829	1301	97.3	50	
	4	4	H1829	1308	98.98	67.53	
	4	4	H1829	1314	98.75	82.28	
11/28/2018	4	5	H1824	1206	95.43	48.5	Note 3
	4	5	H1824	1301	94.87	48.65	
	4	5	H1824	1308	97.33	70.88	
	4	5	H1829	1314	96.93	41.77	
	4	5	H1829	1206	98.39	43.81	
	4	5	H1829	1301	99.03	46.08	
	4	5	H1829	1314	94.24	43.33	

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Assay Date	Season/Cycle	Age of Infection (months)	Donor	Replicate (Fistulated Ewe)	% Viability	% Live Exsheathment	Notes
11/7/2018	5	1	1829	1206	99.03	41.95	
	5	1	1829	1301	98.31	29.55	
	5	1	1829	1308	100	52.43	
	5	1	1829	1314	100	69.31	
	5	1	1833	1206	99.42	52.91	
	5	1	1833	1301	99.09	45.41	
	5	1	1833	1308	98.98	53.09	
	5	1	1833	1314	98.24	43.37	
12/5/2018	5	2	1829	1206	98.94	34.22	
	5	2	1829	1301	99.46	75.41	
	5	2	1829	1308	98.89	58.99	
	5	2	1829	1314	95.33	81.37	
	5	2	1833	1206	96.72	61.02	
	5	2	1833	1301	95.58	83.82	
	5	2	1833	1308	98.3	54.34	
	5	2	1833	1314	100	63.06	
1/7/2019	5	3	1829	1206	99.09	50.69	
	5	3	1829	1301	100	28.06	
	5	3	1829	1308	97.55	86.16	
	5	3	1829	1314	99.05	84.62	
	5	3	1833	1206	99.57	46.09	
	5	3	1833	1301	98.04	68	
	5	3	1833	1308	97.95	71.13	
	5	3	1833	1314	98.34	62.92	
1/30/2019	5	4	1829	1206	97.01	64.19	
	5	4	1829	1301	98.68	85.33	
	5	4	1829	1308	98.7	75.66	
	5	4	1829	1314	98.09	72.82	
	5	4	1833	1206	98.48	67.53	
	5	4	1833	1301	99.42	93.02	
	5	4	1833	1308	99.52	77.99	
	5	4	1833	1314	99.44	84.44	
2/27/2019	5	5	1829	1206	99.52	65.38	
	5	5	1829	1301	98.88	68.93	
	5	5	1829	1308	99.4	52.69	
	5	5	1829	1314	98.29	89.53	
	5	5	1833	1206	98.35	51.4	
	5	5	1833	1301	99.49	49.75	
	5	5	1833	1308	97.01	18.52	
	5	5	1833	1314	98.8	89.69	

Notes	
Note 1	Larvae recovery for one donor failed (due to low viability coming out of culture)
Note 2	Larvae recovery for one donor failed (<95% L3 coming out of culture)
Note 3	Nunc top failure- larvae escaped capsule (NS= no sample)

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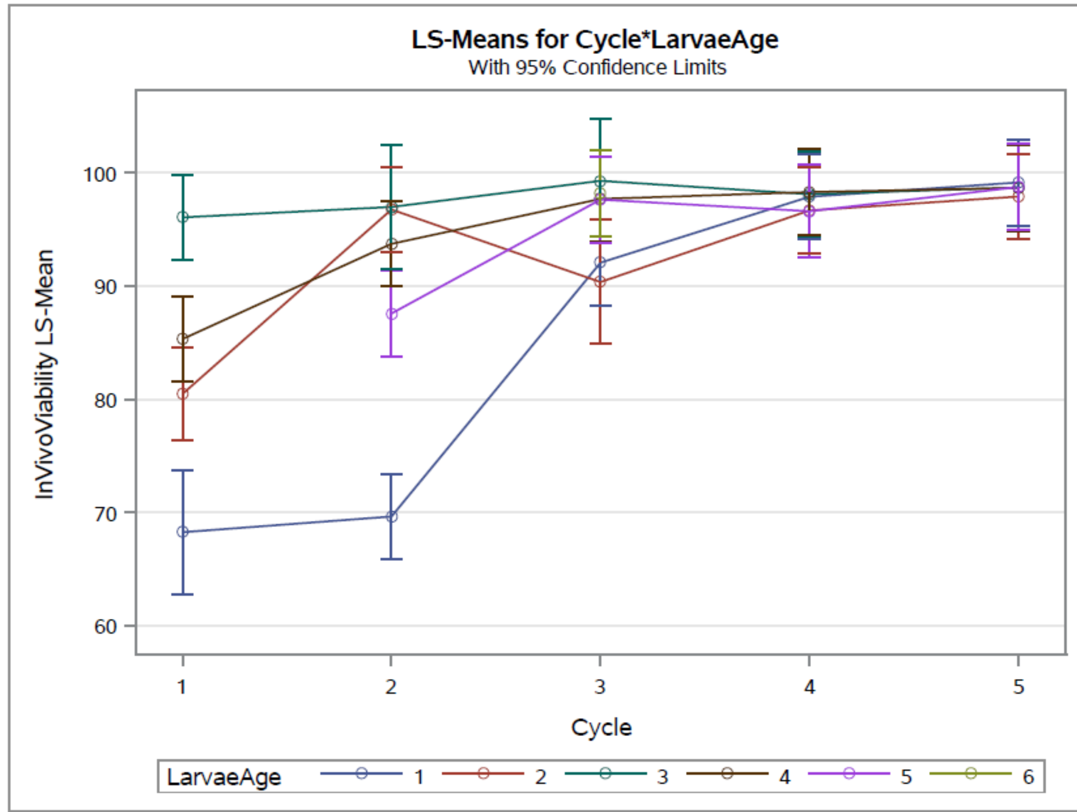
APPENDIX 16

Graph of *In vivo* Exsheathment Assay Viability Results



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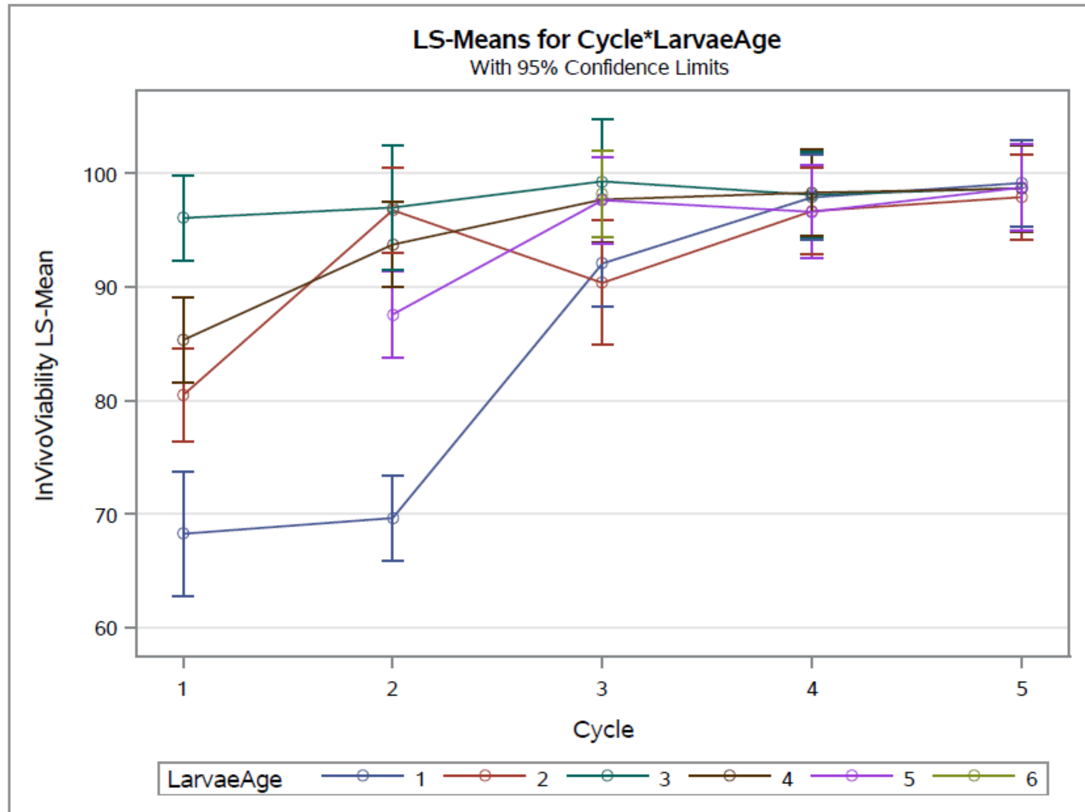
APPENDIX 17

Graph of *In vivo* Exsheathment Assay Exsheathment Results



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APPENDIX 18

Procedure for pH Calibration/Measurement

Supplies:

- shoulder length gloves
- gloves
- pH 7 and 4 buffers
- small cups
- Accumet portable pH meter and electrode (AP115, Fisher Scientific, Hampton, NH)
- large beaker of water
- four small glass beakers
- large syringe with a long piece of Tygon® tubing attached
- record sheet and pen
- distilled water
- halters

Procedure:

1. Attach the electrode to the pH meter (meter should be protected by a gallon zip lock bag).
2. Turn pH meter on by pressing "on" button.
3. With gloves on, remove storage bulb from end of electrode and place the bulb into a cup.
4. Pour approximately 3/4in of each buffer into separate cups.
5. Rinse probe off with distilled water

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6. Press standardize button, and when standardize is flashing on screen, insert into pH 7 buffer.
7. When the pH is stable for 30 seconds, press the standardize button again.
8. Repeat steps 5-7 for pH 4 buffer.
9. Rinse probe and place it in the large beaker of water.
10. Halter and tie sheep to pens.
11. Remove rumen cannula plug and insert the free end of the Tygon® tubing deep into the center of the rumen.
12. Draw back on the syringe to pull rumen fluid into the tubing.
13. Cover the end of the tubing with a finger and remove it from the rumen.
14. Place the end of the tube into one of the clean beakers and release the rumen fluid into the beaker.
15. Take a second sample of rumen fluid from the same ewe and add it to the beaker.
16. Rinse the electrode off with distilled water and place it in the beaker with rumen fluid.
17. Thoroughly rinse the syringe and tubing with water.
18. Record the pH of the rumen fluid.
19. Repeat steps 11-18 on the other ewes.
20. Release ewes and clean up any mess.
21. Rinse the electrode, and with gloves on, reinsert probe into the storage bulb.
22. Dispose of pH buffer into waste containers.

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APPENDIX 19

Fistulated Ewe pH Data for All Cycles

	Fistulated Ewe Identification Number					
Date	1206	1301	1308	1314	Mean	Notes
	pH	pH	pH	pH		
10/31/2017	6.54	6.65	6.49	6.24	6.48	
11/28/2017	6.12	6.05	6.22	5.89	6.07	
12/19/2017	6.14	6.27	6.26	5.95	6.16	
1/23/2018	6.25	6.18	6.25	6.22	6.23	
1/30/2018	6.18	6.22	6.57	5.95	6.23	
2/27/2018	6.12	5.97	6.07	5.61	5.94	
3/27/2018	6.17	6.2	5.97	5.99	6.08	
4/24/2018	NS	NS	NS	NS	N/A	Note 1
5/1/2018	6.83	6.85	6.47	6.53	6.67	
5/22/2018	6.97	6.74	6.96	6.7	6.84	
5/29/2018	6.83	6.54	6.13	6.44	6.49	
7/4/2018	6.72	6.41	6.22	6.18	6.38	
7/31/2018	6.43	NS	6.42	6.17	6.34	Note 2
8/7/2018	6.45	6.44	5.92	6.11	6.23	
8/29/2018	6.8	6.56	6.39	6.28	6.51	
9/5/2018	6.61	6.32	6.31	6.54	6.45	
9/26/2018	6.35	6.36	6.5	6.56	6.44	
10/3/2018	6.24	6.15	6.2	6.31	6.23	
10/30/2018	6.89	6.75	6.81	6.66	6.78	
11/7/2018	6.64	6.41	6.34	6.36	6.44	
11/28/2018	6.71	5.85	6.53	5.57	6.17	
12/5/2018	6.48	6.62	6.68	6.25	6.51	
1/2/2019	7.16	7.28	7.22	7.21	7.22	Note 3
1/30/2019	7.01	6.9	6.81	6.83	6.89	
2/27/2019	6.35	6.55	6.58	6.15	6.41	

	Notes
Note 1	pH meter broken--> no pH measurements taken (NS= no sample)
Note 2	One ewe had rumen fluid issue (NS= no sample)
Note 3	Change in ewes diet to feeding 1x/day instead of 2x/day (for duration of the trial)

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APPENDIX 20

Infection Parameters for All Cycles

Cycle	Infection Start Date	Donor Used for Infection	Age of Donor Infection (Months)	L3 Storage time (Months)	Donors Infected	Donor Gender	Breed	Genetic Relation	Age at time of Infection (Months)	Infection Length (Months)	Culture Period for L3 Harvest	Notes
Fall 1	09/22/17				RI 01-1704	M	Dorset					
		1627	3	< 1	RI 01-1705	M	Dorset	Twins	6	4	Months 1-4: 7 days	
Winter	12/21/17				RI 01-1706	M	Dorset				Months 1-3: 7 days	Note 1 Note 2
		1705	3	< 1	RI 01-1707	M	Dorset	Twins	9	5	Months 4-5: 9 days	Note 3
Spring	03/22/18				Hopkins-1820	F	Dorset Cross				Months 1-2: 9 days	Note 4 Note 5
		1706	2	< 1	Hopkins-1821	F	Dorset Cross	Twins	6	6	Months 3-6: 14 days	Note 6
Summer	06/21/18				Hopkins-1824	F	Dorset Cross	Same Sire				Note 7
		H1820	3	< 1	Hopkins-1829	F	Dorset Cross		9	5	Months 1-5: 14 days	Note 8
Fall 2	09/21/18				RI 01-1829	M	Dorset					Note 8
		H1829	2.5	< 1	RI 01-1833	M	Dorset	Same Sire	6	6	Months 1-6: 14 days	Note 9

Notes	
Note 1	Donor 1705 chosen for infection due to higher L3 viability compared to Donor 1704
Note 2	7 day culture for Donor 1706 failed at 7 days
Note 3	Moved to 9 day culture period for months 4-5 due to 7 day failure in month 3
Note 4	Donor 1706 chosen for infection due to higher concentration of L3 compared to Donor 1707
Note 5	9 day culture failed for Donor H1821 for month 2
Note 6	Moved to 14 day culture period for months 3-6 due to 9 day failure in month 2
Note 7	Donor H1820 chosen for infection due to higher %L3 in larvae harvest compared to donor H1821 (juvenile larvae found in cultures)
Note 8	7 day cultures prepared, flooded and checked for % L3. If not > 95% L3 for both donors, 14 day cultures were used
Note 9	Donor H1829 chosen for infection due to higher %L3 in larvae harvest compared to H1824 (juvenile larvae found in cultures)

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APPENDIX 21

Trial Donor Animal Identification Chart

Animal ID	Gender	Date of Birth	Sire	Sire Registered #	Dam	Dam Registered #	Breed	Notes
RI 01-1704	M	March 9, 2017	PA54-Maple Hollow 1435	P712540 Ram	RI 01-1209	P708498 Ewe	Dorset	
RI 01-1705	M	March 9, 2017	PA54-Maple Hollow 1435	P712540 Ram	RI 01-1209	P708498 Ewe	Dorset	
RI 01-1706	M	March 9, 2017	OHO261-Myers 8866	P717271 Ram	RI 01-1538	P718947 Ewe	Dorset	
RI 01-1707	M	March 9, 2017	OHO261-Myers 8866	P717271 Ram	RI 01-1538	P718947 Ewe	Dorset	
Hopkins-1820	F	September 10, 2017	OHO261-Myers 8866	P717271 Ram	D&D Hopkins 1418	NR	Dorset Cross	Note 1
Hopkins-1821	F	September 10, 2018	OHO261-Myers 8866	P717271 Ram	D&D Hopkins 1418	NR	Dorset Cross	Note 1
Hopkins-1824	F	September 10, 2019	OHO261-Myers 8866	P717271 Ram	D&D Hopkins 1502	NR	Dorset Cross	Note 1
Hopkins-1829	F	September 10, 2019	OHO261-Myers 8866	P717271 Ram	D&D Hopkins 1508	NR	Dorset Cross	Note 1
RI 01-1829	M	April 2, 2018	RI 01-URI 1650	P731125 Ram	RI 01-1608	P724844 Ewe	Dorset	
RI 01-1833	M	April 4, 2018	RI 01-URI 1650	P731125 Ram	RI 01-1506	P718947 Ewe	Dorset	
				Notes				
Note 1	Crossbred ewe (NR= not registered)							

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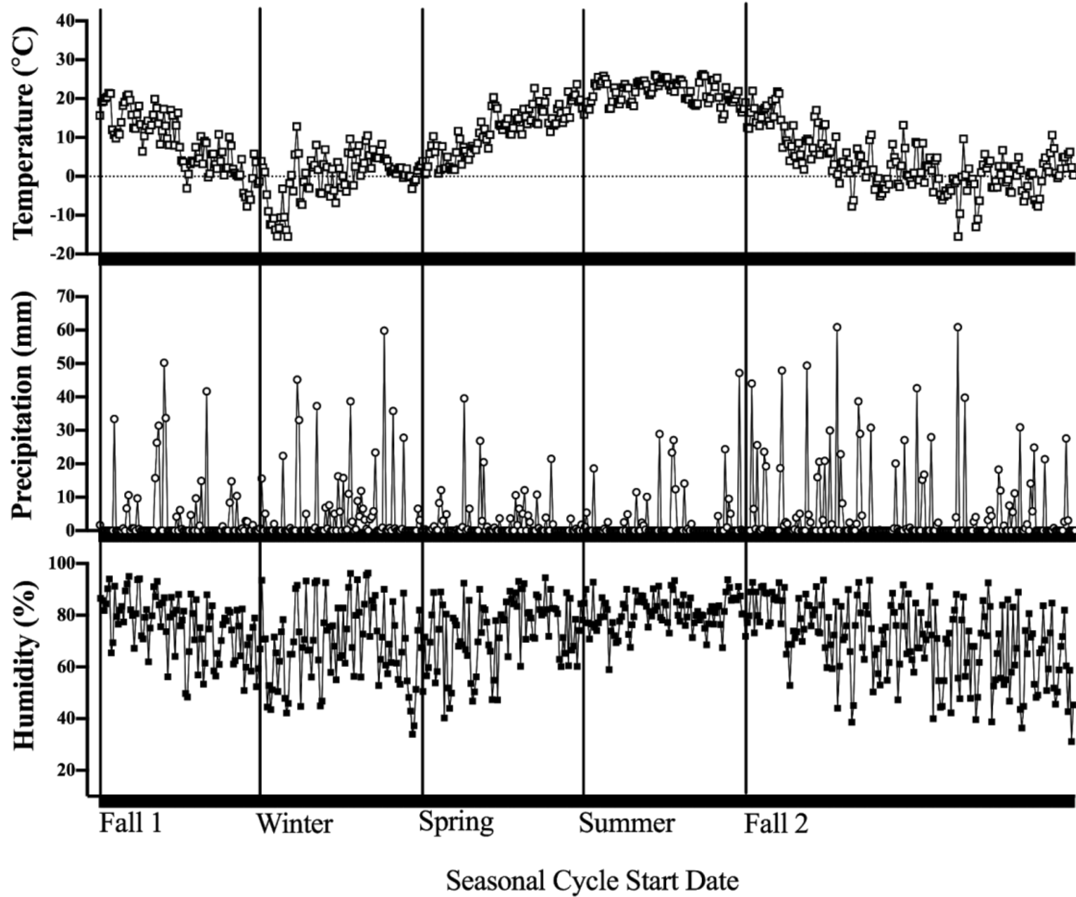
APPENDIX 22

Weather Data



Average daily temperature, precipitation and humidity throughout the five seasonal cycles. Vertical lines designate the start of each seasonal cycle. The length of each cycle, dictated by the persistence of the donor infection, varied from 4-6 months in length, causing sampling within each cycle to extend into the next season. Weather data was collected from an online quality-controlled weather station data set sponsored by NOAA (Weather station title: 'RI Kingston 1 W') (Lawrimore et al., 2011). (https://www.ncdc.noaa.gov/crn/qcdatasets.html).

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Weather Data Description:

Daily average temperature, precipitation and humidity data was collected using a NOAA data set gathered from a weather station in Kingston, RI ('Kingston RI 1 W') (Lawrimore et al., 2011). Average daily temperature, precipitation and humidity for each seasonal cycle are depicted in **Figure 1**. For cycles Fall 1, Winter, Spring, Summer and Fall 2, average cycle temperature was 5.5 ± 0.8°C, 3.6 ± 0.6°C, 16.5 ± 0.5°C, 16.6 ± 0°C, 3.6 ± 0.5°C; average cycle precipitation was 4.2 ± 0.9 mm, 4.3 ± 0.8 mm, 2.9 ± 0.5 mm, 4.9 ± 0.9 mm, 5.5 ± 0.9 mm and average cycle humidity was 73.2 ± 1.2%, 69.7 ± 1.2%, 77.3 ± 0.8%, 79.5 ± 0.8%, 70.1 ± 1.1%, respectively. While average cycle temperature and humidity were both higher for Fall 1 when compared to Fall 2, average cycle precipitation was lower. Weather data was not included in the manuscript of the thesis due to the location of the weather station in relation to the location of the animals used in the study. The weather station is located in a hay field at the University of Rhode Island's Peckham farm and the animals on trial were housed in an indoor space with minimal insulation. Animals were exposed to altered environmental temperatures due to lack of insulation, but were not allowed access to the outdoor environment. The weather station was located approximately 1,150 feet away from the animal housing area. Due to these discrepancies, weather data was not analyzed in this study.

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